JOURNAL OF

Insect Physiology

VOLUME 4 1960

OL.
4
960

EDITORS:

V. G. DETHIER PHILADELPHIA **H. E. HINTON** BRISTOL **M. LÜSCHER** BERN

Editorial Advisory Board

Australia
D. F. WATERHOUSE

France
R. CHAUVIN P. JOLY

Germany
H. AUTRUM K. VON FRISCH B. HASSENSTEIN A. KÜHN

Holland
J. DE WILDE

Italy
C. JUCCI L. PARDI

Japan
S. FUKUDA

Switzerland
E. HADORN

United Kingdom
W. H. THORPE V. B. WIGGLESWORTH F. P. W. WINTERINGHAM

U.S.A.
L. E. CHADWICK A. G. RICHARDS K. D. ROEDER C. M. WILLIAMS

U.S.S.R.
A. S. DANILYEVSKY

PERGAMON PRESS · *OXFORD LONDON NEW YORK PARIS*

Notes for Contributors

I. GENERAL

1. Papers offered for publication should be original contributions in the field of insect physiology or be concerned with similar physiological problems in other groups of arthropods. Anatomical papers and papers on new techniques and methods may be accepted if they contribute to the solution of physiological problems.

2. Papers should be submitted to the appropriate editor:
PROF. V. G. DETHIER *Zoological Laboratory, University of Pennsylvania, Philadelphia* 4, *Pa., U.S.A.*
DR. H. E. HINTON *Department of Zoology, University of Bristol, England*
PROF. M. LÜSCHER *Zoologisches Institut der Universität Bern, Sahlistrasse* 8, *Bern, Switzerland*

3. Fifty free reprints of each paper are supplied. Additional copies may be purchased if they are ordered when the first proofs are returned. A reprint order form will be supplied with the galley proofs.

II. SCRIPT REQUIREMENTS

1. Papers must be typewritten (double spacing) on one side of the paper only. Tables and legends should be on separate sheets, and the place where they are to be inserted in the text should be indicated on the galley proofs. All papers must be provided with a brief abstract. Papers in French or German should be submitted with English abstracts. It will be appreciated if authors clearly indicate any Greek characters. To conserve space, authors are requested to mark less important portions of the papers, such as descriptions of methods, records of experimental results, etc. for printing in smaller type. The technical description of methods should be given in detail only when such methods are new. Authors will receive proofs for correction when their contributions are first set; page proofs will be sent only when the amount of alteration makes it advisable.

2. Illustrations should accompany the script separately, and legends should be kept to a minimum. Line-drawings or good photo-prints may be provided. Line drawings should include all relevant details: if they are already well drawn it may be possible to reproduce them direct from the originals. It is not possible to reproduce from prints with weak lines. Illustrations for reproduction should normally be about twice the final size required. Half-tones should be included only where they are essential. Any lettering on diagrams—other than Greek letters, symbols, etc.—should be stencilled and should be sufficiently large and bold to permit reproduction when the diagram has been reduced to a size suitable for inclusion in the journal.

3. Tables and figures should be constructed so as to be intelligible without reference to the text. Every table should be provided with an explanatory caption and each column should carry an appropriate heading. Units of measure should always be clearly indicated. The same data should not be published in tables and figures. The following standard characters may be used:

▲ △ ⊡ □ ● ○ ◬ ○ ⊕ ⊖ ⊘ ⊗.

4. References should be quoted in the text as follows: EYSENCK (1947). References should be listed at the end of the paper and not given as footnotes. They should be arranged in alphabetical order as follows:

EYSENCK H. J. (1947) *Dimensions of Personality*. Routledge & Kegan Paul, London.
FRENCH J. D., VERZEANO M. and MAGOUN H. W. (1953) A neural basis of the anaesthetic state. *Arch. Neurol. Psychiat.* **69**, 591–600.
WIGGLESWORTH V. B. (1952) The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting. *J. exp. Biol.* **29**, 561–570.

Titles of periodicals cited are to be abbreviated as in the *World List of Scientific Periodicals, 3rd edition*, 1952.

Publishing Offices: Headington Hill Hall, Oxford (*Oxford* 64881)

Issued in parts. Subscription (*A*) *per volume* £7 (*U.S.A.* $20) *including postage;* (*B*) *For subscribers who notify the Publisher direct certifying that the journal is for their private use* £5 5*s.* (*U.S.A.* $15) *per annum including postage.*

Two volumes per annum

Back numbers £8 ($24) *per volume*

Subscriptions should be sent to:
Pergamon Press Ltd., Headington Hill Hall, Oxford; or:
Pergamon Press, Inc., 122 *East* 55*th Street, New York* 22, *N.Y.*

Copyright © 1960 *Pergamon Press Ltd.*

PERGAMON PRESS LTD.

HEADINGTON HILL HALL, OXFORD 122 EAST 55TH STREET, NEW YORK 22, N.Y.

Contents of Volume 4

VOL.
4
1960

Vol. 4, *No.* 1

PAGE

YAMASAKI, T. and NARAHASHI, T.: Synaptic transmission in the last abdominal ganglion of the cockroach 1

FAULKNER, P. and BHEEMESWAR, B.: Metabolism of oximes in the silkworm *Bombyx mori* L.—II. Metabolism of pyruvic oxime $-2^{14}C$ 14

BOCCACCI, M., NATALIZI, G., and BETTINI, S.: Research on the mode of action of halogen containing thiol alkylating agents on insects. Effect of iodoacetic acid on choline acetylase 20

BARTON BROWNE, L. and EVANS, D. R.: Locomotor activity of the blowfly as a function of feeding and starvation 27

NEMENZ, H.: On the osmotic regulation of the larvae of *Ephydra cinerea* 38

ROEDER, K. D., TOZIAN, L., and WEIANT, E. A.: Endogenous nerve activity and behaviour in the mantis and cockroach 45

HOYLE, G.: The action of carbon dioxide gas on an insect spiracular muscle 63

MURRAY, D. R. P.: The stimulus to feeding in larvae of *Tenebrio molitor* L. 80

WAY, M. J.: The effects of freezing temperatures on the developing egg of *Leptohylemyia coarctata* Fall. (Diptera, Muscidae) with special reference to diapause development 92

PAPERS TO BE PUBLISHED IN FUTURE ISSUES 102

Vol. 4, *No.* 2

PASCAL, M. et BAUD, L.: Variabilité de la teneur en phosphore des ovocytes chez *Bombyx mori* L. 103

SIMPSON, J.: The functions of the salivary glands of *Apis mellifera* 107

AUTRUM, H. and HOFFMANN, C.: Diphasic and monophasic responses in the compound eye of *Calliphora* 122

LEWIS, S. E., WALLER, J. B., and FOWLER, K. S.: The effect of DDT and of physically induced prostration on acetylcholine levels in the cockroach 128

BURKHARDT, D.: Action potentials in the antennae of the blowfly (*Calliphora erythrocephala*) during mechanical stimulation 138

PATEL, N. G. and RICHARDS, A. G.: Proteolytic enzymes from the midgut of the housefly 146

LEES, A. D.: The role of photoperiod and temperature in the determination of parthogenetic and sexual forms in the aphid *Megoura viciae* Buckton—II. The operation of the 'interval timer' in young clones 154

HINTON, H. E.: Plastron respiration in the eggs of blowflies 176

WIGGLESWORTH, V. B. and BEAMENT, J. W. L.: The respiratory structures in the eggs of higher Diptera 184

PAPERS TO BE PUBLISHED IN FUTURE ISSUES 190

Vol. 4, *No.* 3

BECHT, G., HOYLE, G., and USHERWOOD, P. N. R.: Neuromuscular transmission in the coxal muscles of the cockroach 191

HUOT, L. et VERLY, W. G.: Dosage du ^{32}P total d'une larve vivante de *Tenebrio molitor* L. (Coléoptère: Tenebrionidae) 202

MILES, P. W.: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera: Lygaeidae)—II. Physical and chemical properties 209

PAGE

PARK, H. D. and BUCK, J.: The relation of oxygen consumption to ambient oxygen concentration during metamorphosis of the blowfly, *Phormia regina* 220

SHYAMALA, M. B., SHARADA, K., BHAT, M. G., and BHAT, J. V.: Chloromycetin in the nutrition of the silkworm, *Bombyx mori* L.—II. Influence on digestion and utilization of protein, fat, and minerals 229

SELMAN, B. J.: On the tissue isolated in some of the larval appendages of *Sialis lutaria* L. at the larval–pupal moult 235

HOWDEN, G. F. and KILBY, B. A.: Biochemical studies on insect haemolymph—I. Variations in reducing power with age and the effect of diet 258

PAPERS TO BE PUBLISHED IN FUTURE ISSUES 270

Vol. 4, *No.* 4

MILES, P. W.: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera: Lygaeidae)—III. Origins in the salivary glands 271

LANGER, H.: Die Wirkung von Licht auf den chemischen Grundaufbau des Auges von *Calliphora erythrocephala* Meig. 283

BECK, S. D. and HANEC, W.: Diapause in the European corn borer, *Pyrausta nubilalis* (Hübn.) 304

DADD, R. H.: The nutritional requirements of locusts—I. Development of synthetic diets and lipid requirements 319

NEWBURGH, R. W., POTTER, L. N., and CHELDELIN, V. H.: Oxidative phosphorylation in *Phormia regina* larvae 348

PAPERS TO BE PUBLISHED IN FUTURE ISSUES 350

Author Index

AUTRUM H. p. 122
BARTON BROWNE L. p. 27
BAUD L. p. 103
BEAMENT J. W. L. p. 184
BECHT G. p. 191
BECK S. D. p. 304
BETTINI S. p. 20
BHAT J. V. p. 229
BHAT M. G. p. 229
BHEEMESWAR B. p. 14
BOCCACCI M. p. 20
BUCK J. p. 220
BURKHARDT D. p. 134
CHELDELIN V. H. p. 348
DADD R. H. p. 319
EVANS D. R. p. 27
FAULKNER P. p. 14
FOWLER K. S. p. 128
HANEC W. p. 304
HINTON H. E. p. 176
HOFFMANN C. p. 122
HOWDEN G. F. p. 258
HOYLE G. pp. 63, 191
HUOT P. p. 202
KILBY B. A. p. 258
LANGER H. p. 283
LEES A. D. p. 154
LEWIS S. E. p. 128
MILES P. W. pp. 209, 271
MURRAY D. R. P. p. 80
NARAHASHI T. p. 1
NATALIZI G. p. 20
NEMENZ H. p. 38
NEWBURGH R. W. p. 348
PARK H. D. p. 220
PASCAL M. p. 103
PATEL N. G. p. 146
POTTER L. N. p. 348
RICHARDS A. G. p. 146
ROEDER K. D. p. 45
SELMAN B. J. p. 235
SHARADA K. p. 229
SHYAMALA M. B. p. 229
SIMPSON J. p. 107
TOZIAN L. p. 45
USHERWOOD P. N. R. p. 191
VERLY W. G. p. 202
WALLER J. B. p. 128
WAY M. J. p. 92
WEIANT E. A. p. 45
WIGGLESWORTH V. B. p. 184
YAMASAKI T. p. 1

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 1 *to* 13. *Pergamon Press Ltd., London. Printed in Great Britain*

SYNAPTIC TRANSMISSION IN THE LAST ABDOMINAL GANGLION OF THE COCKROACH

TERUO YAMASAKI and TOSHIO NARAHASHI

Laboratory of Applied Entomology, Faculty of Agriculture,
University of Tokyo, Tokyo, Japan

(*Received* 18 *December* 1958)

Abstract—Excitatory postsynaptic potentials (e.p.s.p.'s) were recorded from the last abdominal ganglion of the cockroach. The e.p.s.p. was much increased and prolonged by anticholinesterases, and such a change in e.p.s.p. was found to be responsible for the prolonged synaptic after-discharge. It was found that inactivation of cholinesterase runs parallel with these changes in electrical responses under the influence of anticholinesterases. The sensitivity of the ganglion to applied acetylcholine was increased by desheathing and eserinization. Three possible explanations for the nature of transmitter substance were suggested; the possibility of acetylcholine as transmitter still remains.

VOL.
4
1960

SINCE fundamental studies by PUMPHREY and RAWDON-SMITH (1937), ROEDER (1948b), and ROEDER *et al.* (1947), synapses across the last abdominal ganglion of the cockroach have been used for examining the effects of drugs and insecticides such as anticholinesterases. Despite these studies knowledge seems to be less complete than that of synapses in vertebrate animals. For example, in sympathetic ganglia of vertebrates, liberation of transmitter substance from the presynaptic nerve terminals, depolarization of the postsynaptic membrane by the transmitter substance, and discharge of propagated impulses from this depolarization have been extensively studied. Synaptic events in the central nervous system of vertebrates have also been extensively explored, although the transmitter substance is not yet identified in most synapses (cf. ECCLES, 1953, 1957). Since most insecticides are nerve poisons, more detailed analyses of the synaptic transmission in insects are required. It is the purpose of this study to analyse the electrical phenomena associated with synaptic transmission and to clarify the nature of the synaptic transmitter in the last abdominal ganglion of the cockroach.

METHODS

Insects

Adults of the American cockroach, *Periplaneta americana* L., reared in the laboratory at a temperature of about 30°C were used throughout the experiments.

Nerve preparations

The synaptic pathway across the last (sixth) abdominal ganglion was studied in detail by ROEDER (1948b) and ROEDER *et al.* (1947). For electrophysiological study, the nerve

preparation, which included the cercus, the cercal nerve, the abdominal nerve cord, and the third thoracic ganglion, was isolated from the cockroach.

Stimulation and recording

Condenser discharges having a time constant of about 0·25 msec or thyratron discharges intervening an inductorium were applied to the peripheral end of the cercal (presynaptic) nerve through a pair of fine silver wire electrodes. Presynaptic responses were recorded from the cercal nerve just before entering the ganglion with the aid of a pair of fine silver wire or of fine silver–silver chloride wire electrodes. Postsynaptic responses were recorded at the origin of the connectives from the last abdominal ganglion; an indifferent electrode was in contact with the third thoracic ganglion which had usually been crushed by forceps to make the recorded action potentials monophasic. For recording the postsynaptic responses fine silver–silver chloride wire electrodes were used in the initial stage of the present study, whereas the same type of zinc–zinc sulphate electrodes as described in our previous paper (YAMASAKI and NARAHASHI, 1957) was used in the later stages of the work. A CR-coupled amplifier having a time constant of about 1 sec was used at first, whereas a d.c. amplifier was used in most later experiments.

Solution and drugs

The Ringer's solution used contained 159·6 mM Na^+, 3·1 mM K^+, 1·8 mM Ca^{2+}, 160·1 mM Cl^-, 0·2 mM $H_2PO_4^-$, and 1·8 mM HPO_4^{2-}, and showed pH of 7·2. Eserine sulphate, tetraethylpyrophosphate (TEPP), and ethyl parathion were used as anticholinesterases. Parathion was so purified that IN50 for cholinesterase was of the order of 10^{-8}. In order to find out the agent that blocks synaptic transmission selectively, phenobarbital, ether, phenothiazine, rotenone, N-(3′-dimethylaminopropyl)-3-chlorophenothiazine hydrochloride (Contomin), 2-mercaptotropone, and urethane were tested. Acetylcholine chloride (ACh) was also used. Parathion, phenothiazine, and rotenone were applied as suspensions which had been prepared by introducing drug acetone or alcohol into a Ringer's solution. Ether was applied as a saturated solution or as a gas. The other drugs were directly dissolved in Ringer's solution, the pH being carefully adjusted at an initial value of 7·2.

All the electrophysiological experiments were conducted at room temperatures ranging from 14°C to 32°C. The temperature during any single series of experiments did not change by more than 1°C.

Determination of cholinesterase (ChE)

ChE determinations of the nerve cord were made by estimating the ACh content using Hestrin's colorimetric method (1949). A nerve cord was homogenized after adding a buffer solution containing 0·15 M NaCl, 0·04 M $MgCl_2$, and 0·025 M $NaHCO_3$ and showing pH of 7·4, the concentration of homogenate being 7 or 8 mg wet weight per millilitre of buffer solution. A 0·5 ml sample of homogenate was incubated with 0·5 ml of 0·016 M ACh for 30 min at 37°C. After incubation the quantity of the remaining ACh was estimated colorimetrically.

RESULTS

A. Normal synaptic transmission

A series of pre- and the corresponding postsynaptic responses is shown in Fig. 1 (a, b). Increasing the stimulus strength caused the presynaptic response to increase in spike height. In the postsynaptic response, however, the spike height markedly increased only in the initial responses. These initial large postsynaptic action potentials can safely be assumed to originate in the giant axons, which have been described by ROEDER (1948b) and ROEDER *et al.* (1947).

With higher amplification, however, additional features became apparent. In the first place a slow potential was observed at threshold stimulation (Fig. 1 c). The slow potential had a rising phase of 2–3 msec and decayed exponentially with a time constant of 5–20 msec. When the stimulus strength was increased above threshold, one or a few spike potentials were generated from the summit of the slow potential. These spike potentials formed the initial large postsynaptic responses originating in the giant axons. When the different electrode was moved along the connectives far from the last abdominal ganglion, the slow potential decreased in height and slowed its time course. The longer the distance from the last abdominal ganglion the smaller was the height of the slow potential and the slower was its time course. Comparing the oscillograms of the slow potential with those of the presynaptic response revealed that the slow potential was generated somewhat later than the presynaptic response.

All these features make it quite reasonable to assume that the slow potential is a so-called excitatory postsynaptic potential (e.p.s.p.) electrotonically transmitted along the ascending axons to the origin of the connectives. This will hereafter be termed the 'initial e.p.s.p.' or simply the 'e.p.s.p.'

VOL. 4 1960

A small monophasic action potential was observable immediately before the onset of the e.p.s.p. (Fig. 1 d). The small action potential progressively increased in height in parallel with the presynaptic response as the stimulus strength was increased. Even though synaptic transmission was blocked by such drugs as anticholinesterase, the small action potential as well as the presynaptic action potential was still observable. Comparing the oscillograms of the small action potential with those of the presynaptic response revealed that the onset of the small action potential nearly corresponded to the initial peak of the diphasic presynaptic action potential (Fig. 1 d). It follows that the onset of the small action potential coincides with the time of arrival of an impulse at the origin of the cercal nerve, because one of the electrodes for recording the presynaptic responses is actually in contact with this point. On this evidence the small action potential recorded postganglionically is considered to be the presynaptic action potential transmitted electrotonically across the ganglion.

Another slow potential associated with the discharges was observable 30–50 msec after the initial postsynaptic response (Fig. 1 e). It can be assumed that this represents a polysynaptic ganglionic response. This will hereafter be termed the 'delayed e.p.s.p.'

Synaptic delay. By measuring the interval between the onset of the small action potential recorded postganglionically and the onset of the e.p.s.p., and by subtracting the conduction time of the preganglionic fibres in the ganglion from this measurement, synaptic delay can be measured (Fig. 1 d). Assuming the conduction velocity of the cercal nerve to be 1·5–2 m/sec (ROEDER, 1948b; ROEDER *et al.*, 1947), the conduction time along 0·2 mm of nerve amounts to 0·1 msec. From these measurements an average synaptic delay of 1·59 msec (0·97–2·08 msec) is obtained by calculation.

B. Effects of anticholinesterases on synaptic transmission

Some time (10–40 min) after applying 10^{-5} eserine to the nerve, both the initial and the delayed e.p.s.p.'s are increased and prolonged, and the frequency of discharges superimposed upon them is also increased (Fig. 2 a). In parallel with these changes in synaptic transmission, even with the absence of stimulation, the discharges of small axons increased in frequency and the slow wave of small amplitude increased both in frequency and in magnitude. It was found that the discharges did not change in frequency even after amputating the cercal nerve.

The increases in e.p.s.p. and in after-discharge became more apparent with the advance of time. The initial e.p.s.p. grew so marked that the discrimination between the initial and the delayed e.p.s.p.'s became obscure, forming a long-lasting slow potential (depolarization) upon which a prolonged after-discharge was superimposed (Fig. 2 a). The e.p.s.p. was augmented and prolonged more and more, its rising or depolarizing phase occupying from several seconds to 10 sec and its falling or repolarizing phase as long as 3 min. At this stage, however, the after-discharge showed a special pattern (Fig. 2 b, c). The after-discharge immediately following the initial response ceased as the depolarization of the ganglion reached its greatest extent. It started again following repolarization, and then ceased when repolarization was almost complete. This observation suggests the possibility that impulses are discharged at a critical level of depolarization.

After further advance of time, usually 30–90 min after applying eserine, however, the prolonged e.p.s.p. did not return to the original repolarized level, and synaptic transmission remained blocked. The depolarization of the ganglion continued from several minutes to as long as 20 min.

Although recovery from the depolarization did not always occur, a gradual repolarization occurred in most cases. Washing with saline accelerated the recovery. In the course of recovery, the impulses of the giant axons were discharged without stimulation when the repolarization reached a critical level, and ceased following complete repolarization. Full recovery generally took from several minutes to 10 min.

The presynaptic response, however, remained unchanged during the course of this sequence of events. Hence it can safely be concluded that the synapses or the postsynaptic elements are responsible for the appearance of ganglionic block as well as the after-discharge.

In the course of recovery from the persistent depolarization, the ganglion cells discharged impulses in response to a presynaptic volley, and the size of the response grew more and more in parallel with the progress of repolarization (Fig. 2 f).

A similar type of persistent depolarization associated with prolonged discharges was often observed in the absence of presynaptic stimulation. In this case also a restoration was usually brought about after a period of depolarization (Fig. 2 d, e). Furthermore, the sequence of depolarization-after-discharge (or burst)-block-restoration could be repeated without further application of eserine or even after washing with saline.

VOL.
4
1960

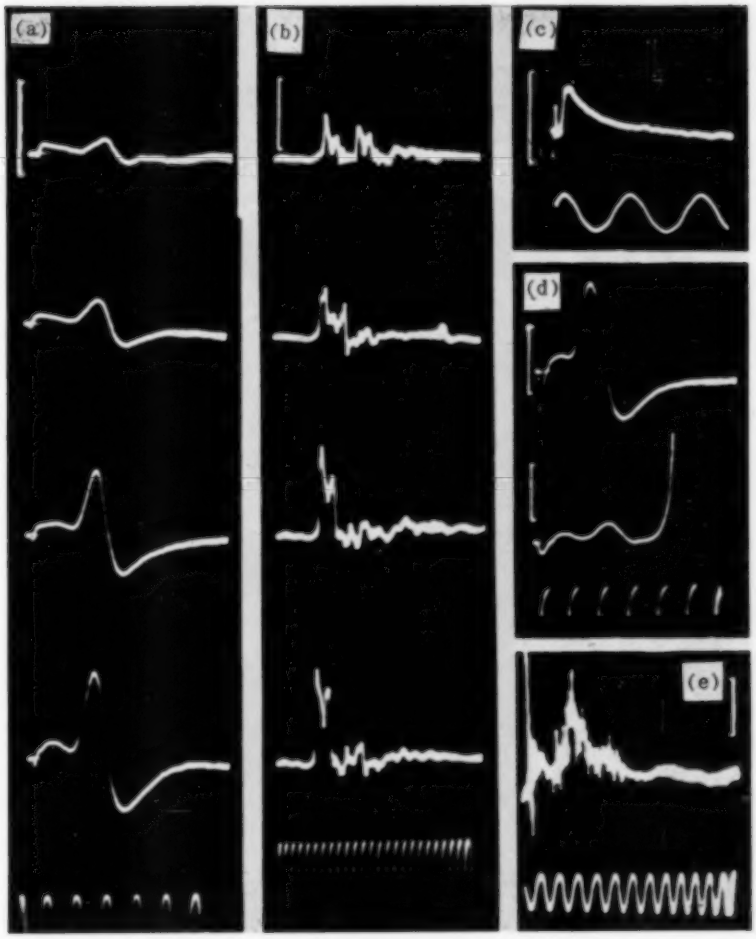


FIG. 1. (a) Presynaptic responses. Stimulus strength was increased from top to bottom. Voltage calibration, 10 mV. Time marker, 1000 c/sec. 16·5°C. (b) Postsynaptic responses corresponding to column (a). Voltage calibration, 3 mV. Time marker, 1000 c/sec. (c) An e.p.s.p. set up by a weak presynaptic volley. Voltage calibration, 1 mV. Time marker, 50 c/sec. 21·5°C. (d) Upper record, a presynaptic response. Voltage calibration, 3 mV. Lower record, a postsynaptic response showing an electrotonically transmitted presynaptic action potential and a rising phase of the postsynaptic action potential. Voltage calibration, 0·5 mV. Time marker, 1000 c/sec. 16·5°C. (e) A postsynaptic response showing the delayed e.p.s.p. superimposed with discharges. Voltage calibration, 0·5 mV. Time marker, 50 c/sec. 16·5°C.

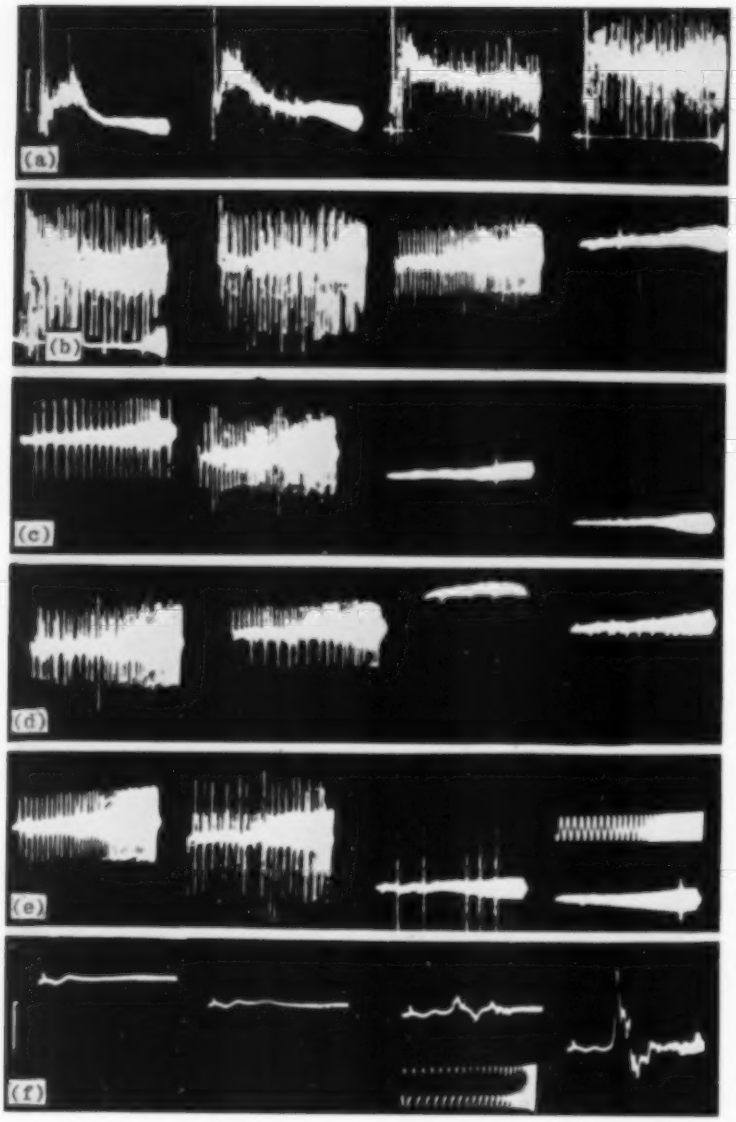


FIG. 2. Effects of anticholineaterases on the ganglion. (a) Postsynaptic responses. From left to right, before, 12, 19, and 29 min after treatment with 5×10^{-5} eserine, respectively. 14°C. (b) As in (a), but 56 min after, showing a prolonged depolarization and discharge. All records are arranged on a same base line shown in the left record. From left to right, responses at a time of presynaptic stimulation, 15, 30, and 45 sec after stimulation, respectively. (c) As in (b), but 60, 75, 90, and 180 sec after stimulation. (d) and (e) Spontaneous discharges, 81 min after treatment with 5×10^{-5} eserine. All records are arranged on a same base line. The time intervals between successive records are 15, 30, 60, 30, 15, 45, and 15 sec, respectively. A voltage calibration of 0·5 mV shown in (a) and a time marker of 50 c/sec shown in (e) apply to (a–e). (f) Postsynaptic responses showing the dependence of synaptic transmission on the membrane potential; 240 min after treatment with 10^{-5} parathion. All records are arranged on a same base line. The time intervals between successive records are 2·5, 2, and 6 min, respectively. Voltage calibration, 5 mV. Time marker, 1000 c/sec. 14°C.

VOL. 4 1960

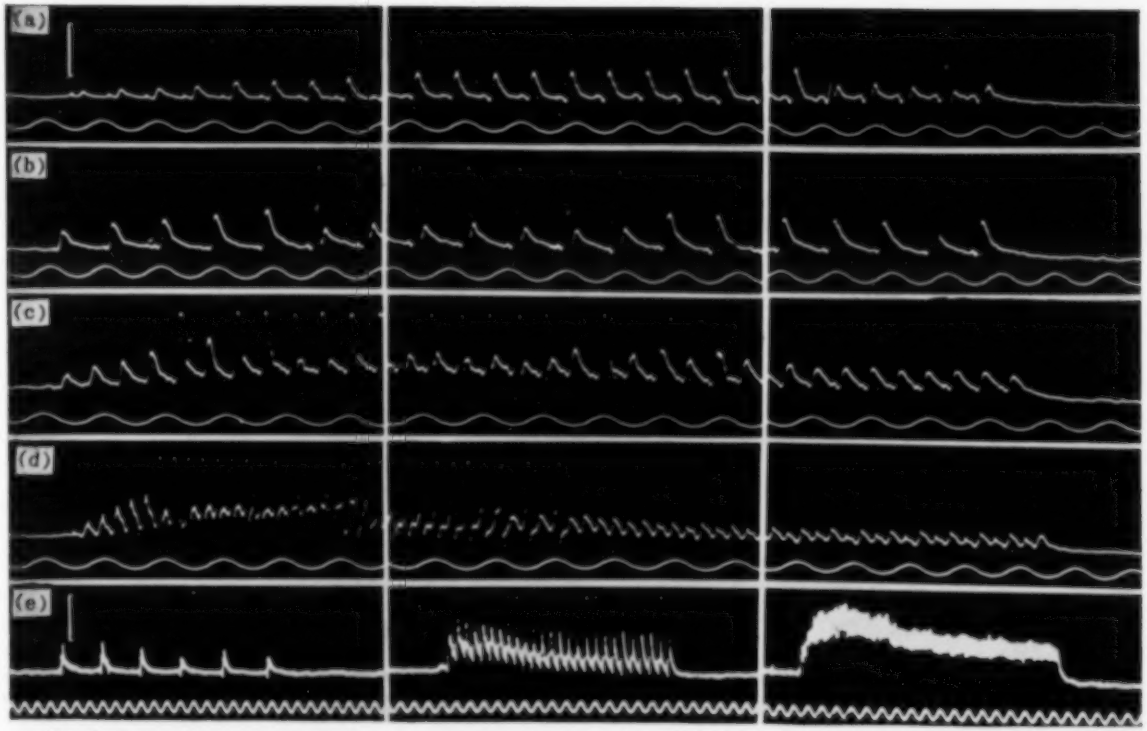


FIG. 3. E.p.s.p.'s set up by repetitive presynaptic volleys. Under 5×10^{-4} phenobarbital. (a) By weak stimuli. Action potentials, shown by dots, are generated at the latest part of the record. The time intervals between successive records are 110 msec. A voltage calibration of 1 mV applies to (a–d). Time marker, 50 c/sec. 20·5°C. (b) As in (a), but by stronger stimuli. Action potentials are generated earlier. The time intervals between successive records are 160 and 70 msec respectively. (c) As in (b), but by repetitive stimuli with higher frequency. Action potentials appear much earlier. The time intervals are 100 and 180 msec respectively. (d) As in (c), but by much higher frequency. The time intervals are 90 and 120 msec respectively. (e) Another preparation. E.p.s.p.'s set up by repetitive volleys of different frequency. Voltage calibration, 0·5 mV. Time marker, 50 c/sec. 30°C.

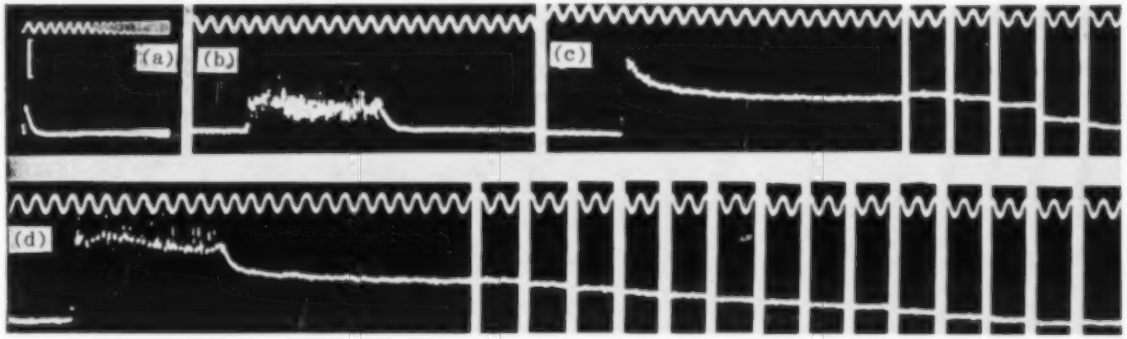


FIG. 4. Effect of eserine on the e.p.s.p. Under 5×10^{-2} urethane. 22°C. (a) Before treatment. An e.p.s.p. set up by a single presynaptic volley. A voltage calibration of 0·5 mV applies to all records. Time marker, 50 c/sec. (b) As in (a), but by repetitive volleys. Time marker, 50 c/sec. (c) 26 min after treatment with 10^{-5} eserine. An e.p.s.p. set up by a single volley. The time intervals between successive records are 400 msec except for the last one, which is 260 msec. Time marker, 50 c/sec. (d) As in (c), but 32 min after and by repetitive volleys. The time intervals between successive records are 460 msec except for the last one, which is 100 msec. Time marker, 50 c/sec.

With 10^{-4} eserine depolarization and synaptic block were brought about much earlier; the depolarization and after-discharge being induced 1–4 min after application, and the synaptic block 3–20 min after application. Furthermore with 10^{-4} eserine a restoration was seldom brought about without washing.

The effect of 10^{-5} or 10^{-6} TEPP was the same as that of eserine in most respects. It blocked synaptic transmission 8–30 min after application.

Parathion (10^{-5}) had almost the same effect though the time course of sequence was much prolonged. After-discharge became apparent 45–130 min after application, and synaptic block was caused 70–220 min after application. Since parathion itself is not a strong inhibitor of ChE and is converted in animals to paraoxon which strongly inhibits ChE (METCALF and MARCH, 1953), a long latent period for the appearance of after-discharge is likely to be attributed to the time necessary for the conversion of parathion in the nerve. Recovery from the persistent depolarization and block was also brought about without washing.

VOL. 4 1960

C. Attempts to record the pure e.p.s.p. using drugs

Of all detectable changes in the postsynaptic membrane, it is the e.p.s.p. which is most closely related to synaptic transmission. The e.p.s.p. recorded by the present technique, however, is complicated by superimposed action potentials. This was also the case with the neuromuscular junction and the synapses of sympathetic ganglia or spinal cord in vertebrates, but the difficulty could be overcome by employing pharmacological agents such as curarine and nembutal (ECCLES, 1943, 1946; KUFFLER, 1942). However, since curare has no strong blocking action on insect ganglia (ROEDER *et al.*, 1947), attempts were made to find suitable drugs for depressing postsynaptic discharges.

Ether, rotenone (10^{-5} or 10^{-6}), and N-(3′-dimethylaminopropyl)-3-chlorophenothiazine hydrochloride (Contomin, 10^{-3}) caused a progressive conduction block of the presynaptic fibres. With 2-mercaptotropone ($1{\cdot}33 \times 10^{-4}$) synaptic transmission was not impaired, though the e.p.s.p. was slightly increased. Phenothiazine (10^{-4}) was without effect both on the presynaptic conduction and on the synaptic transmission. Therefore, these drugs could not be used for observing the pure e.p.s.p.

Phenobarbital (10^{-3}) and urethane ($1–5 \times 10^{-2}$) caused a selective synaptic block. The presynaptic conduction was partly and progressively blocked when these drugs were allowed to be in contact with the nerve after the synaptic block had occurred. Washing with saline brought about a complete recovery. Therefore, by employing suitable concentrations of these drugs, it was possible to observe the pure e.p.s.p. for a long time.

D. Summation of e.p.s.p.'s

When a series of weak repetitive volleys was applied to the preganglionic nerve under the influence of phenobarbital or urethane, a temporal summation could be observed (Fig. 3). At the onset of repetitive stimulation there was a brief phase of potentiation during which the successive e.p.s.p.'s were increased

to several times their initial values. This potentiation eventually caused the e.p.s.p.'s to reach a critical level and hence to discharge impulses. Therefore, it can be said that the summation is effective in relieving a blockage of synaptic transmission, as with curare-blockage of neuromuscular transmission in vertebrates (ECCLES *et al.*, 1941). The stronger the stimulus strength or the higher the stimulus frequency, the earlier was the e.p.s.p. from which impulses were generated (Fig. 3 a–d). The summed e.p.s.p.'s, however, decreased in height after reaching a peak (Fig. 3 b–d). Therefore, the impulses, being once generated by temporal summation, might fail to be generated if repetitive stimulation was continued.

On cessation of repetitive stimulation the summed e.p.s.p. decayed exponentially, the time course of decay being not significantly different from that of the e.p.s.p. evoked by a single volley (Fig. 3 e). There was little or no sign of after-positivity.

E. Effects of anticholinesterases on e.p.s.p.

Although it has been shown in Section *B* that anticholinesterases such as eserine, TEPP, and parathion cause the ganglion to give a very prolonged e.p.s.p. superimposed with after-discharges, there is still the possibility that the prolonged e.p.s.p. is due to the summation of high frequency discharges. This problem could be solved by observing the pure e.p.s.p.

Eserine (10^{-5}) was applied to the nerve showing the pure e.p.s.p. under urethane (Fig. 4 a, b). The e.p.s.p. increased in size and its declining phase became prolonged after a time. Eventually the declining phase continued for as long as 3 sec (Fig. 4 c). With repetitive stimulation the e.p.s.p. might continue for as long as 10 sec (Fig. 4 d).

F. Cholinesterase activity

Six separate determinations of ChE activity resulted in a mean value of 1·36 μM ACh hydrolysed per milligramme wet weight of nerve cord per hour at 37°C.

The ChE activity was considerably different in each nerve cord. The next series of experiments was done to see whether a great difference in ChE activity could be found between the anterior and the posterior regions of a nerve cord. The results are shown in Table 1, in which the differences in ChE activity between the anterior and the posterior regions of nerve cords are shown to be relatively small.

TABLE 1—ChE ACTIVITY IN THE ANTERIOR AND THE POSTERIOR REGIONS OF NERVE CORDS (μM ACh split per milligramme cord per hour at 37°C)

Preparation	First and second thoracic ganglia	Third thoracic and abdominal ganglia
A	1·51	1·12
B	1·25	1·09
C	1·12	1·11
D	1·27	1·42
E	1·61	1·37

G. Nervous activity and inactivation of cholinesterase

In view of the evidence described in the foregoing section, the anterior region of a nerve cord, including the first and second thoracic ganglia, was used as a control while the posterior region was treated with anticholinesterases. When prolongation of after-discharge became apparent after dosage with 2×10^{-5} or 3×10^{-5} parathion, the nerve preparation was immediately homogenized and the activity of ChE was determined. The percentages of ChE inhibition are given in Table 2. It was found in another set of experiments that ChE was completely inhibited in a nerve in which synaptic transmission had been blocked. Although fluctuations of the results shown in Table 2 are considerable, it can be said that the inactivation of ChE runs parallel with the synaptic after-discharge.

TABLE 2—PER CENT INHIBITION OF ChE IN THE NERVE DEVELOPED SYNAPTIC AFTER-DISCHARGE FOLLOWING DOSAGE WITH PARATHION

No.	Temperature of parathion treatment (°C)	Concentration of parathion	Period of parathion treatment (min sec)	Per cent inhibition
1	16	3×10^{-5}	18.00	3
2	16	3×10^{-5}	20.00	28
3	16	3×10^{-5}	12.00	41
4	16	3×10^{-5}	12.30	57
5	17	3×10^{-5}	14.00	0
6	17	3×10^{-5}	17.15	18
7	17	2×10^{-5}	18.45	23
8	17	2×10^{-5}	18.15	37
9	17	2×10^{-5}	13.15	42

H. Effect of acetylcholine on the intact and desheathed ganglion

Marked increase and prolongation of the e.p.s.p. after treatment with anticholinesterases strongly suggests the possibility that ACh is a transmitter substance in the last abdominal ganglion, but in order to establish the ACh theory it is necessary to demonstrate that applied ACh can stimulate the ganglion cells to discharge impulses.

At a concentration of 10^{-3} or less ACh was without effect either on synaptic transmission or on spontaneous discharge when applied to the intact ganglion. With 10^{-2} ACh the ganglion discharged impulses. Both the giant axons and small axons discharged impulses within 1 min after application. The discharge continued for about 5–20 min, and was followed by a transient high-frequency burst and a synaptic block. Following pre-treatment with 10^{-5} eserine, 10^{-3} ACh became effective in causing the intact ganglion to discharge impulses.

Acetylcholine (10^{-3}) had a striking stimulating action on the desheathed ganglion, and although 10^{-4} ACh had little effect on it, a pre-treatment with 10^{-5} eserine made it effective in discharging impulses (Fig. 5); 10^{-5} ACh had little effect on the eserinized desheathed ganglion.

VOL. 4 1960

In order to study the effect of pre-treatment with eserine it was of importance to apply ACh to the ganglion in the after-discharge condition. Furthermore, it was also necessary to eliminate mechanical stimulation caused by ACh application, because the eserinized ganglion was very sensitive to any kind of stimulation. In the present experiments the drug-containing saline was applied by a pipette to the ganglion, which was mounted on electrodes. With such precautions the following procedure was adopted. Eserine (10^{-5}) was initially applied to the ganglion. When the effect of the eserine became apparent to such an extent that the synaptic after-discharge was considerably prolonged, the normal saline was gently applied. This washing has usually no mechanical effect. The washing with saline was repeated once or twice, thus it was confirmed that applications of saline had no effect on the ganglion. Following these procedures ACh was applied to the ganglion.

DISCUSSION

After treatment with anticholinesterases, discharges are initiated at a critical range of ganglionic depolarization, they cease when the depolarization exceeds this range, and are initiated again on the way to repolarization. Furthermore, synaptic transmission is blocked when the depolarization exceeds this critical range. These changes in ganglionic membrane potential are likely to be responsible for the cyclic alternation of recovery and block observed with anticholinesterases by TWAROG and ROEDER (1957).

In vertebrates, there is now convincing evidence that ACh is a synaptic transmitter in sympathetic ganglia as well as at neuromuscular junctions. In order to confirm the role of ACh as synaptic transmitter the following should be demonstrated experimentally: (1) The preganglionic fibres contain a store of ACh and are capable of synthesizing ACh. (2) The preganglionic impulses cause the liberation of ACh from the preganglionic terminals. (3) Low concentrations of ACh are effective in depolarizing the ganglion cells and discharging impulses from them. (4) ChE is concentrated on the preganglionic fibres or within the ganglion cells. (5) Inactivation of ChE is effective in increasing and prolonging the e.p.s.p.'s and evoking the prolonged after-discharge from them. These requirements will be discussed in sequence as they apply to the cockroach ganglion.

1. *Synthesis and store of* ACh. The thoracic nerve cord of the cockroach contains as much ACh as 45 μg/g and is capable of synthesizing ACh (TOBIAS *et al.*, 1946). This figure for ACh content is 3–30 times as large as that for vertebrate sympathetic ganglia or brains (BROWN and FELDBERG, 1936; CHANG and GADDUM, 1933; TOBIAS *et al.*, 1946). Comparable values for ACh content in the cockroach nerve cord are also given by several investigators, i.e. 36·7 μg/g by LEWIS and SMALLMAN (1956), 70 μg/g by MIKALONIS and BROWN (1941) and 32 μg/g by ROEDER (1948a). Furthermore, the ACh content of the sixth abdominal ganglion has recently been estimated to be 63·0 μg/g (COLHOUN, 1958a). However, these investigators estimated ACh content by bioassay. Therefore it remains to be decided whether the active substance responsible for the test is ACh or is a substance that is not identical with,

VOL.
4
1960

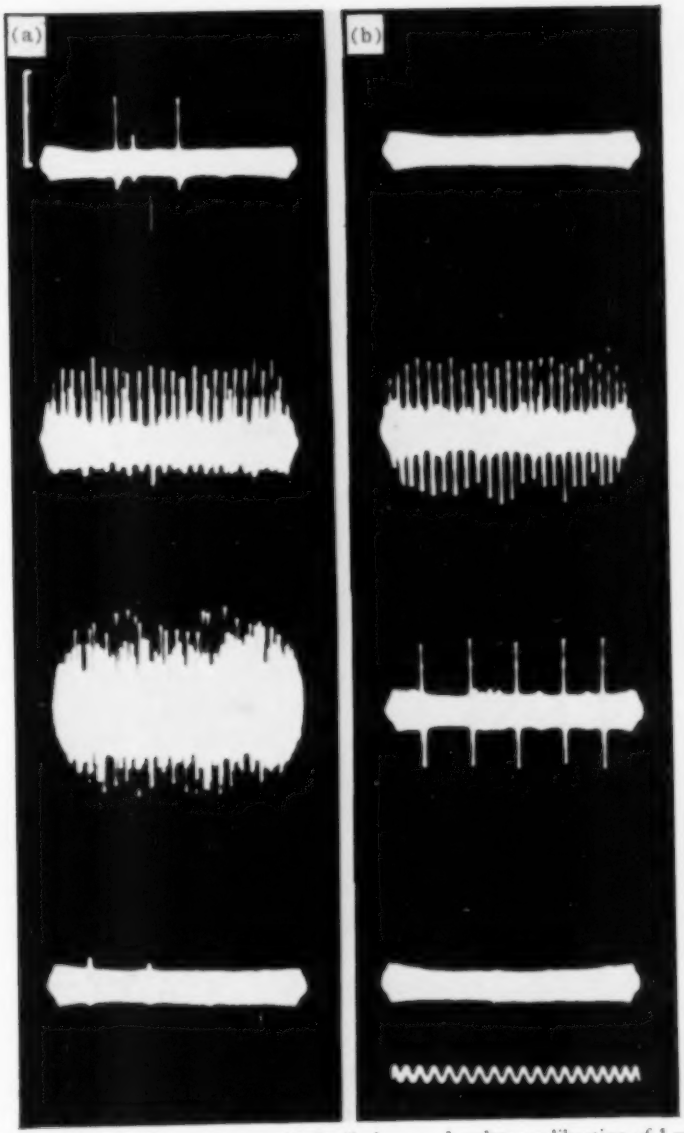


FIG. 5. Effect of ACh on the ganglionic discharge. A voltage calibration of 1 mV and a time marker of 50 c/sec apply to all records. Only discharges are recorded by a CR-amplifier. 23° C. (a) A desheathed and eserinized (10^{-5}) ganglion, showing a burst followed by a block after treatment with ACh. From top to bottom, 30 sec after treatment with saline, 10, 30, and 40 sec after treatment with 10^{-4} ACh, respectively. (b) Another desheathed and eserinized ganglion, showing a transient discharge followed by a recovery after treatment with ACh. From top to bottom, 30 sec after treatment with saline, 10, 20, and 120 sec after treatment with 10^{-4} ACh, respectively.

but only resembles ACh. Furthermore, it should be pointed out that the detection of ACh in the cercal nerve is indispensable for establishing the ACh theory in the synapses across the last abdominal ganglion. For these reasons the knowledge obtained up to date is insufficient for concluding that transmission is cholinergic in the last abdominal ganglion.

2. *Liberation of* ACh. Although a number of exhaustive studies have been performed with good success using the vertebrate sympathetic ganglia (e.g. FELDBERG and GADDUM, 1934; FELDBERG and VARTIAINEN, 1935; MACINTOSH, 1938), few data are available for insect ganglia. COLHOUN (1958b) has recently shown that electrical stimulation, when applied to the connectives between the fifth and sixth abdominal ganglia of the roach, causes an increase in ACh content in the eserinized ventral cord. This result, however, does not contribute directly to the present problem, because it is highly desirable to demonstrate the liberation of ACh from the terminals of the cercal nerve fibres when they are stimulated. COLHOUN (1958b) has further shown that when the cerci are stimulated by air puffs no appreciable difference is detected in ACh content between the normal and stimulated sixth abdominal ganglia. It seems reasonable to assume that the amount of ACh needed for synaptic transmission is so minute that no difference in ACh level is detectable. Despite all these studies, insufficient data are available for solving the present problem.

3. *Action of* ACh. Only a very high concentration of ACh (10^{-2}) is effective in discharging impulses from the intact cockroach ganglion. When the ganglion is desheathed and is eserinized, however, ACh becomes effective in concentrations as low as 10^{-4}, although 10^{-5} ACh still has little effect. The threshold concentration of ACh is therefore of the same order of magnitude as that found by TWAROG and ROEDER (1957) with the eserinized desheathed ganglion, i.e. 10^{-4} M. In the vertebrate sympathetic ganglia, however, where ACh certainly plays the role of synaptic transmitter, the threshold concentration of ACh is as low as 5×10^{-7} when its ChE has been inactivated (EMMELIN and MACINTOSH, 1952). In vertebrate skeletal muscle, ACh is also effective in depolarizing the end-plate and in generating impulses in concentration as low as 10^{-6} (KUFFLER, 1943, 1945). Therefore, the threshold concentration of ACh in the cockroach ganglion is about 100 times as high as those in the sympathetic ganglia and in the end-plate.

4. *Cholinesterase*. ChE is concentrated on the preganglionic fibres or within the ganglion cells in sympathetic ganglia of vertebrates (KOELLE, 1951; NACHMANSOHN, 1940). ChE is also found in the brain of mammals, the activity usually ranging from 0·2 to 0·5 μM ACh hydrolysed per milligramme wet weight per hour (MCILWAIN, 1955; METCALF and MARCH, 1950). The ChE activity of the roach nerve cord as revealed by the present experiments is considerably higher than that of mammals, the mean value being 1·36 μM ACh/mg wet weight/hr at 37°C. The same order of activity, 31–32 mg ACh/100 mg wet weight/hr at 37·6°C, was reported with the roach thoracic ganglia (TOBIAS *et al.*, 1946). Histochemical analysis has revealed the dense localization of ChE on the surface of neurones both in ganglia and in connectives (IYATOMI and KANEHISA, 1958).

VOL. 4 1960

5. *Effect of cholinesterase inactivation.* Inactivation of ChE by treatment with anticholinesterases is effective in increasing and prolonging the e.p.s.p.'s and/or evoking a prolonged after-discharge from the nerve cells in the vertebrate sympathetic ganglia as well as in skeletal muscles (e.g. BROWN *et al.*, 1936; ECCLES, 1944; ECCLES and MACFARLANE, 1949; ECCLES *et al.*, 1942; FATT and KATZ, 1951). This has been shown to be the case in the cockroach as described in this paper. A prolongation of the e.p.s.p. following treatment with anticholinesterases seems to be explicable as follows: an inactivation of ChE by anticholinesterases allows the synaptic transmitter to act on the subsynaptic membrane for some time and hence the e.p.s.p. becomes prolonged. It follows that the transmitter is an ester which can be hydrolysed by ChE.

Under such considerations there are three possible explanations concerning the nature of the synaptic transmitter in the last abdominal ganglion of the cockroach. In the first place, although low effectiveness of ACh in depolarizing the roach ganglion and in discharging impulses makes it somewhat difficult to assume ACh as synaptic transmitter, the possibility cannot be excluded. If the susceptibility of the subsynaptic membrane to the synaptic transmitter were low, it would be necessary for the transmitter to be liberated in a large amount and for the hydrolysing enzyme to be highly concentrated. In fact ACh has a weak synaptic action in the cockroach and the ChE activity is high. Hence the possibility of ACh as transmitter cannot be excluded.

In the second place, the weak synaptic action of ACh may be explained on the basis of a physical barrier preventing access to the synapses. In fact sheath-like structures have been noted around the individual giant axons and certain groups of neurones in the cockroach (TWAROG and ROEDER, 1956). These structures may prohibit effectively the penetration of applied ACh to the subsynaptic membrane. This possibility makes it less improbable that ACh is a synaptic transmitter.

In the third place, the synaptic transmitter may not be ACh but be an ACh-like substance. It is reasonable to suppose that the transmitter is hydrolysed by ChE, for inactivation of the ChE in the ganglion causes the e.p.s.p. to be augmented and prolonged; it suggests a persistent action of the transmitter.

The mode of blocking action of the anaesthetics, phenobarbital, and urethane, stands in striking contrast to that of anticholinesterases. With these anaesthetics no depolarization is observed, but the discharge of impulses from the e.p.s.p. is effectively depressed. At a later stage of poisoning, however, the size of e.p.s.p. is progressively diminished. It seems likely therefore that these anaesthetics block synaptic transmission largely by increasing the stability of the ganglion cell membrane, and also partly by diminishing the height of the e.p.s.p. There is general agreement that most anaesthetics have a similar stabilizing action (BROOKS and ECCLES, 1947; ECCLES, 1946; SHANES, 1948).

SUMMARY

(1) Synaptic transmission across the last abdominal ganglion of the cockroach has been studied by recording both local and propagated responses from the

ganglion with and without various drugs, and by determining the cholinesterase (ChE) activity of the nerve cord.

(2) A presynaptic volley sets up excitatory postsynaptic potentials (e.p.s.p.'s). When the e.p.s.p. reaches a certain critical level, action potentials of the giant axons are generated.

(3) The mean value of synaptic delay was 1·59 msec.

(4) Delayed e.p.s.p.'s with superimposed discharges of small axons were observed, with a latency of about 30–50 msec.

(5) It was found that phenobarbital and urethane are effective in prohibiting the discharge from ganglion cells leaving the pure e.p.s.p. These drugs are considered to stabilize the nerve cell membrane.

(6) At the onset of weak repetitive stimulation there was a brief phase of potentiation during which impulses were discharged. A depression followed and persisted for a long time. There was no sign of prolonged decay or after-positivity of the e.p.s.p. on cessation of repetitive stimulation.

(7) An application of anticholinesterases such as eserine, TEPP, and parathion caused a marked increase and prolongation of the e.p.s.p.'s superimposed with a prolonged after-discharge. The e.p.s.p.'s grew so marked that a persistent ganglionic depolarization associated with synaptic block was eventually brought about. A repolarization associated with recovery of the synaptic transmission occurred after some time. It was found that it is not the presynaptic but the postsynaptic elements of the synapses which are responsible for these events.

(8) The ChE activity of the nerve cord was determined as 1·36 μM acetylcholine (ACh) hydrolysed per milligramme wet weight of nerve cord per hour at 37°C.

(9) Inactivation of ChE runs parallel with the after-discharge.

(10) When applied to the intact ganglion, the threshold concentration of ACh for discharging impulses was as high as 10^{-2}. With the eserinized or desheathed ganglion, it was lowered to 10^{-3}. When the desheathed ganglion was eserinized, it was further lowered to 10^{-4}.

(11) The nature of the transmitter substance in the last abdominal ganglion of the cockroach was discussed in the light of the present results, and three possible explanations were suggested. The possibility of ACh as transmitter still remains.

VOL. 4 1960

REFERENCES

BROOKS C. McC. and ECCLES J. C. (1947) A study of the effects of anaesthesia and asphyxia on the mono-synaptic pathway through the spinal cord. *J. Neurophysiol.* **10**, 349–360.

BROWN G. L. and FELDBERG W. (1936) The action of potassium on the superior cervical ganglion of the cat. *J. Physiol.* **86**, 290–305.

BROWN G. L., DALE H. H., and FELDBERG W. (1936) Reactions of the normal mammalian muscle to acetylcholine and to eserine. *J. Physiol.* **87**, 394–424.

CHANG H. C. and GADDUM J. H. (1933) Choline esters in tissue extracts. *J. Physiol.* **79**, 255–285.

COLHOUN E. H. (1958a) Acetylcholine in *Periplaneta americana* L.—I. Acetylcholine levels in nervous tissue. *J. Ins. Physiol.* **2**, 108–116.

COLHOUN E. H. (1958b) Acetylcholine in *Periplaneta americana* L.—II. Acetylcholine and nervous activity. *J. Ins. Physiol.* **2**, 117–127.

ECCLES J. C. (1943) Synaptic potentials and transmission in sympathetic ganglion. *J. Physiol.* **101**, 465–483.

ECCLES J. C. (1944) The nature of synaptic transmission in a sympathetic ganglion. *J. Physiol.* **103**, 27–54.

ECCLES J. C. (1946) Synaptic potentials of motoneurones. *J. Neurophysiol.* **9**, 87–120.

ECCLES J. C. (1953) *The Neurophysiological Basis of Mind.* Clarendon Press, Oxford.

ECCLES J. C. (1957) *The Physiology of Nerve Cells.* Johns Hopkins Press, Baltimore.

ECCLES J. C. and MACFARLANE W. V. (1949) Action of anticholinesterases on endplate potential of frog muscle. *J. Neurophysiol.* **12**, 59–80.

ECCLES J. C., KATZ B., and KUFFLER S. W. (1941) Nature of the "endplate potential" in curarized muscle. *J. Neurophysiol.* **4**, 362–387.

ECCLES J. C., KATZ B., and KUFFLER S. W. (1942) Effect of eserine on neuromuscular transmission. *J. Neurophysiol.* **5**, 211–230.

EMMELIN N. G. and MACINTOSH F. C. (1952) Cited in ECCLES (1953).

FATT P. and KATZ B. (1951) An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

FELDBERG W. and GADDUM J. H. (1934) The chemical transmitter at synapses in a sympathetic ganglion. *J. Physiol.* **81**, 305–319.

FELDBERG W. and VARTIAINEN A. (1935) Further observations on the physiology and pharmacology of a sympathetic ganglion. *J. Physiol.* **83**, 103–128.

HESTRIN S. (1949) The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J. biol. Chem.* **180**, 249–261.

IYATOMI K. and KANEHISA K. (1958) Localization of cholinesterases in the American cockroach. *Jap. J. appl. Ent. Zool.* **2**, 1–10.

KOELLE G. B. (1951) The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol.* **103**, 153–171.

KUFFLER S. W. (1942) Electric potential changes at an isolated nerve–muscle junction. *J. Neurophysiol.* **5**, 18–26.

KUFFLER S. W. (1943) Specific excitability of the endplate region in normal and denervated muscle. *J. Neurophysiol.* **6**, 99–110.

KUFFLER S. W. (1945) Electric excitability of nerve–muscle fibre preparations. *J. Neurophysiol.* **8**, 77–87.

LEWIS S. E. and SMALLMAN B. N. (1956) The estimation of acetylcholine in insects. *J. Physiol.* **134**, 241–256.

MACINTOSH F. C. (1938) Liberation of acetylcholine by the perfused superior cervical ganglion. *J. Physiol.* **94**, 155–169.

MCILWAIN H. (1955) *Biochemistry and the Central Nervous System.* J. and A. Churchill, London.

METCALF R. L. and MARCH R. B. (1950) Properties of acetylcholine esterase from the bee, the fly and mouse and their relation to insecticide action. *J. econ. Ent.* **43**, 670–676.

METCALF R. L. and MARCH R. B. (1953) Further studies on the mode of action of organic thionophosphate insecticides. *Ann. ent. Soc. Amer.* **46**, 63–74.

MIKALONIS S. J. and BROWN R. H. (1941) Acetylcholine and cholinesterase in the insect central nervous system. *J. cell. comp. Physiol.* **18**, 401–403.

NACHMANSOHN D. (1940) On the physiological significance of choline esterase. *Yale J. Biol. Med.* **12**, 565–589.

PUMPHREY R. J. and RAWDON-SMITH A. F. (1937) Synaptic transmission of nervous impulses through the last abdominal ganglion of the cockroach. *Proc. roy. Soc.* (B) **122**, 106–118.

ROEDER K. D. (1948a) The effect of anticholinesterases and related substances on nervous activity in the cockroach. *Johns Hopk. Hosp. Bull.* **83**, 587–603.

ROEDER K. D. (1948b) Organization of the ascending giant fibre system in the cockroach (*Periplaneta americana*). *J. exp. Zool.* **108**, 243–261.

ROEDER K. D., KENNEDY N. K., and SAMSON E. A. (1947) Synaptic conduction to giant fibres of the cockroach and the action of anticholinesterases. *J. Neurophysiol.* **10**, 1–10.

SHANES A. M. (1948) An experimental and theoretical approach to the mechanism of cocaine action. *Science* **107**, 679–681.

TOBIAS J. M., KOLLROS J. J., and SAVIT J. (1946) Acetylcholine and related substances in the cockroach, fly and crayfish and the effect of DDT. *J. cell. comp. Physiol.* **28**, 159–182.

TWAROG B. M. and ROEDER K. D. (1956) Properties of the connective tissue sheath of the cockroach abdominal nerve cord. *Biol. Bull., Woods Hole* **111**, 278–286.

TWAROG B. M. and ROEDER K. D. (1957) Pharmacological observations on the desheathed last abdominal ganglion of the cockroach. *Ann. ent. Soc. Amer.* **50**, 231–237.

YAMASAKI T. and NARAHASHI T. (1957) Studies on the mechanism of action of insecticides —XV. Effects of metabolic inhibitors, potassium ions and DDT on some electrical properties of insect nerve. *Botyu-Kagaku* **22**, 354–367.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 14 *to* 19. *Pergamon Press Ltd., London. Printed in Great Britain*

METABOLISM OF OXIMES IN THE SILKWORM *BOMBYX MORI* L.—II. METABOLISM OF PYRUVIC OXIME $-2^{14}C$*

P. FAULKNER and B. BHEEMESWAR†

Laboratory of Insect Pathology
Sault Ste. Marie, Ontario, Canada

(*Received* 19 *January* 1959)

Abstract—Pyruvic oxime $-2^{14}C$ and pyruvate $-2^{14}C$ were injected into larvae of the silkworm, *Bombyx mori* L. The label in pyruvate $-2^{14}C$ is incorporated into the gut and body proteins, but the small amount of activity found in protein following injection of pyruvic oxime $-2^{14}C$ is of a much lower order and does not increase or decrease with time. $^{14}CO_2$ is expired following injection of pyruvate $-2^{14}C$, whereas the activity in CO_2 after injection of pyruvic oxime $-2^{14}C$ is negligible. Chromatographic analysis of aqueous extracts of faecal pellets following injection or feeding of pyruvic oxime $-2^{14}C$ indicates that it is rapidly excreted unchanged and is not converted to pyruvate $-2^{14}C$. The results of these experiments suggest that transoximase and oximase, if they are present, are of little importance in the protein and carbohydrate metabolism of the silkworm.

INTRODUCTION

EVIDENCE presented by BHEEMESWAR and FAULKNER (1959) indicates that several oximes of naturally occurring keto-acids and carbohydrates are inhibitors of transaminases. It has been postulated by YAMAFUJI and co-workers that in the silkworm, *Bombyx mori* L., oximes are intermediates in the conversion of inorganic nitrogenous compounds to organic amino compounds (YAMAFUJI, YOSHIHARA, and WADA, 1950; YAMAFUJI, KAWAKAMI, and SHINOHARA, 1952). They presented evidence that oximase is involved in the formation of amino compounds from such substances as pyruvic oxime, glucoseoxime, and acetoxime. In order to assess the importance of oximes in the metabolism of the silkworm, we have prepared pyruvic oxime $-2^{14}C$ and have made some observations on its fate following injection and feeding. It was shown by FUKUDA (1957) that when pyruvate $-2^{14}C$ is fed to larval silkworms the label is incorporated into the silk and the activity is found predominantly in the alanine moiety. This is to be expected as glutamic–alanine transaminase has been demonstrated in silkworm tissues (FUKUDA, 1957). Similarly, if pyruvic oxime is converted into pyruvate by transoximase (YAMAFUJI, 1953) or by a hydrolytic enzyme, it would be anticipated that the label in pyruvic oxime $-2^{14}C$ would also be found in proteins. In the present studies we have therefore compared the metabolism of pyruvate $-2^{14}C$ with that of pyruvic oxime $-2^{14}C$.

* Contribution No. 496, Division of Forest Biology, Science Service, Department of Agriculture, Ottawa, Canada.

† National Research Council of Canada, Postdoctoral Fellow.

MATERIALS AND METHODS

Sodium pyruvate – $2^{14}C$ was obtained from Merck and Co., Montreal.

Pyruvic oxime – $2^{14}C$ was prepared as follows: 1·735 mg sodium pyruvate – $2^{14}C$, with activity of 0·05 mc, and 25 mg sodium pyruvate (Nutritional Biochemicals Co., Cleveland, Ohio) were dissolved in 0·5 ml of water, 100 mg sodium acetate and 100 mg hydroxylamine hydrochloride were added, and the mixture was warmed to 80°C on a water bath for 10 min. It was chilled in a refrigerator and crystals appeared on seeding with a little unlabelled pyruvic oxime. The product was re-crystallized from 95 per cent ethanol and had a m.p. of 183–184°C.

The purity of the product was checked by ascending paper chromatography in a solvent composed of amyl acetate : 80 per cent formic acid : water, 4:1:1 by volume (YAMAFUJI and ETO, 1953). The position of the oxime was determined by spraying the paper with 1·0 per cent ferric chloride in absolute ethanol. Pyruvic oxime has an *Rf* value of 0·55 in this solvent while pyruvate remains at the origin. When the preparation of pyruvic oxime – $2^{14}C$ was chromatographed, the radioactivity was found exclusively in the pyruvic oxime position, none being present at the origin, indicating no contamination by pyruvate – $2^{14}C$. A further check on the purity of the pyruvic oxime – $2^{14}C$ preparation was obtained by spectrophotometric analysis for free pyruvate in the presence of reduced diphosphopyridine nucleotide and lactic dehydrogenase. Under our assay conditions, no decrease in optical density occurred at 340 mμ when 2μm of pyruvic oxime – $2^{14}C$ were incubated in the test system, whereas 0·1μm pyruvate added subsequently led to a decrease of 0·150 optical density units.

Insects used in these studies were early fifth instar larvae of the silkworm *Bombyx mori* L.

Injection and feeding experiments. Early fifth instar larvae to be used for injection and feeding experiments were starved overnight. Usually 10μl of a neutralized solution of the substance under test was injected into the tip of a leg using a microsyringe fitted with a glass capillary (BERGOLD, 1941).

In the feeding experiments, small squares (2 cm) of mulberry leaves were coated with the test solution using methyl cellulose as a sticker (ANGUS, 1954).

Analytical methods. Protein was determined by the method of LOWRY *et al.* (1951).

$^{14}CO_2$: When it was necessary to collect $^{14}CO_2$, the larvae were placed in Petri dishes (15 cm × 2 cm) fitted with ground glass tops and sealed with high vacuum grease. A small glass cup, 2 cm diameter and 1·8 cm high, which contained 0·2 ml saturated barium hydroxide, was placed in the centre of the Petri dish. At the end of the $^{14}CO_2$ collection period the centre cup was removed and to it was added 0·1 ml of 0·5 M Na_2CO_3 and sufficient water to transfer the contents to tubes for centrifugation. After centrifugation the $Ba^{14}CO_3$ was suspended in 0·2 ml water and duplicate aliquots of 0·05 ml were transferred to previously weighed stainless steel planchets. The plates were dried for at least 16 hr over silica gel in a vacuum desiccator before the activity was determined in a Tracerlab SC–16 flow counter.

VOL. 4 1960

Isolation of the protein for ^{14}C incorporation studies

At the stated intervals following injection or feeding of pyruvate – $2^{14}C$ or pyruvic oxime – $2^{14}C$, groups of four larvae were bled by snipping the tip off one of the legs. The blood was immediately frozen for storage. The bled larvae were then dissected to remove the guts and the silk glands which were separated and dropped into 5 ml of 6 per cent trichloracetic acid (TCA). The remainder of the larva, termed 'body', was also dropped into 5 ml of 6 per cent TCA.

In order to remove adsorbed radioactive material, the tissues were washed by centrifugation five times with 6 per cent TCA, and a further five times with 6 per cent TCA containing 0·1 per cent unlabelled sodium pyruvate or 0·1 per cent unlabelled pyruvic oxime, depending on whether pyruvate – $2^{14}C$ or pyruvic oxime – $2^{14}C$ had been injected. The tissue was then washed twice with hot 5 per cent TCA (80°C for 15 min) in order to remove nucleic acid, and four times with an ethanol–chloroform mixture (3:1 v/v) to remove lipids. The residual protein was suspended in 1·0 ml water and usually 0·05 ml aliquots were plated in duplicate on steel planchets.

RESULTS

Incorporation of pyruvate – $2^{14}C$ and pyruvic oxime – $2^{14}C$ into protein

Pyruvate – $2^{14}C$ and pyruvic oxime – $2^{14}C$ were injected into groups of four insects and after intervals of 3, 30, and 60 min, the larvae were bled and dissected and the incorporation of the label into the protein fractions of the blood, gut, and 'body' was determined. Both the pyruvate – $2^{14}C$ and the pyruvic oxime – $2^{14}C$ used for injection were prepared in 0·125 M DL-glutamate solution since preliminary work indicated that a higher initial rate of incorporation was obtained under these conditions. The data in Table 1 indicate that the initial radioactivity of the whole blood of the insects was of the same order whether pyruvate – $2^{14}C$

TABLE 1—PROTEIN LABELLING AFTER INJECTION OF PYRUVATE – $2^{14}C$

Tissue	Interval after injection (min)	c/min/10μl whole blood following injection of: pyruvate – $2^{14}C$	pyruvic oxime – $2^{14}C$
Whole blood	3	5557	4286
	30	2877	521
	60	1674	420
Gut protein		cpm/mg protein following injection of: pyruvate – $2^{14}C$	pyruvic oxime – $2^{14}C$
	3	126	53
	30	733	55
	60	424	68
Body protein			
	3	113	61
	30	—	58
	60	950	60

Groups of four insects were injected with 10 μl of either pyruvate – $2^{14}C$ or pyruvic oxime – $2^{14}C$ prepared in 0·125 M DL-glutamate. At the stated intervals the insects were bled and the activity of whole blood, gut, and 'body' protein was determined.

or pyruvic oxime – $2^{14}C$ was injected. The blood ^{14}C level fell slowly during the experimental period following injection of pyruvate – $2^{14}C$, but there was a considerable drop in activity during the first 30 min after pyruvic oxime – $2^{14}C$ injection.

During the experimental period of 60 min, there was no detectable incorporation of the label in pyruvate or pyruvic oxime into the blood protein. However, protein obtained from the gut and 'body' was found to be labelled following injection of pyruvate – $2^{14}C$, but after pyruvic oxime – $2^{14}C$ injection, the level of radioactivity in protein was low and did not show significant change with time during the experimental period (Table 1).

Respired $^{14}CO_2$ after injection of pyruvate – $2^{14}C$ and pyruvic oxime – $2^{14}C$

The data given in Table 2 show that oxidation of pyruvate – $2^{14}C$ to $^{14}CO_2$ takes place rapidly following injection. $^{14}CO_2$ evolved following pyruvic oxime – $2^{14}C$ injection is negligible and indicates that little oxidation of pyruvic oxime takes place.

VOL. 4 1960

TABLE 2—$^{14}CO_2$ FOLLOWING INJECTION OF PYRUVATE – $2^{14}C$ AND PYRUVIC OXIME – $2^{14}C$

Interval after injection (hr)	c/min/mg $Ba^{14}CO_3$ after injection of:	
	sodium pyruvate – $2^{14}C$	pyruvic oxime – $2^{14}C$ sodium salt
0–2·5	450	11
2·5–5·0	207	16
5·0–22·0	112	2

Two groups of five fifth instar silkworm which had been starved for 16 hr were injected with 10 μl sodium pyruvate – $2^{14}C$ (15 μgm salt with total activity 820,000 cpm). The second group received 10μl sodium salt of pyruvic oxime 2 – ^{14}C (130 μgm with total activity 506,000 cpm). $^{14}CO_2$ was collected as described under 'Methods'.

^{14}C content of faecal pellets after injection and feeding of pyruvate – $2^{14}C$ and pyruvic oxime – $2^{14}C$

Faecal pellets collected after the injection of pyruvate – $2^{14}C$ or pyruvic oxime – $2^{14}C$ are found to be radioactive. The data given in Table 3 illustrate that

TABLE 3—^{14}C CONTENT OF FAECAL PELLETS FOLLOWING INJECTION OF PYRUVATE – $2^{14}C$ AND PYRUVIC OXIME – $2^{14}C$

Collection interval (hr)	c/min/mg faecal pellets following injection of:	
	pyruvate – $2^{14}C$	pyruvic oxime – $2^{14}C$
0–5	6300	41,800
5–22	2020	114

Groups of insects were injected with sodium pyruvate – $2^{14}C$ or pyruvic oxime – $2^{14}C$ as described in Table 2. The insects were starved in the interval 0–5 hr following injection, but were fed mulberry leaves during the remaining period. Five pellets were suspended in 0·4 ml water and 0·05 ml was plated onto previously weighed planchets.

pyruvic oxime – $2^{14}C$ is excreted more than six times faster than pyruvate – $2^{14}C$ during the first 5 hr after injection, suggesting that a selective mechanism leads to the retention of pyruvate but pyruvic oxime is cleared rapidly. In order to determine the form in which the ^{14}C is present after injection of pyruvate – $2^{14}C$ or pyruvic oxime – $2^{14}C$, supernatants obtained from aqueous extracts of the faecal pellets were chromatographed with unlabelled pyruvate or unlabelled pyruvic oxime in Yamafuji's solvent. The positions of pyruvate and pyruvic oxime were detected with the ferric chloride reagent and the substances were eluted with 60 per cent methanol and plated. The data given in Table 4 illustrate

TABLE 4—DISTRIBUTION OF ^{14}C IN FAECAL EXTRACT FOLLOWING PYRUVATE – $2^{14}C$ AND PYRUVIC OXIME – $2^{14}C$ INJECTION

	c/min/20 μl faecal pellet extract after injection of:	
	pyruvate – $2^{14}C$	pyruvic oxime – $2^{14}C$
Eluate from origin (pyruvate position)	164	15
Eluate from pyruvic oxime position	14	876

Faecal pellets were those obtained in the 0–5 hr interval in the experiment described in Table 3. Five pellets were extracted in 0·4 ml water and 20 μl supernatant was chromatographed in the amyl acetate–formic acid solvent (YAMAFUJI *et al.*, 1953). Spots were eluted in 0·4 ml 60 per cent methanol and plated.

that, after injection of pyruvate – $2^{14}C$, the activity remains at the origin (the position occupied by pyruvate), but that on treatment with pyruvic oxime – $2^{14}C$ the faecal extract contains pyruvic oxime – $2^{14}C$ but no pyruvate – $2^{14}C$. This is further evidence that neither hydrolysis of pyruvic oxime nor transoximation occurs in the silkworm.

TABLE 5—^{14}C CONTENT OF FAECAL PELLETS AFTER FEEDING PYRUVIC OXIME – $2^{14}C$

Collection period (Hours after feeding pyruvic oxime – $2^{14}C$)	c/min/mg pellets	c/min/20μl faecal pellet extract: eluate from origin pyruvate position	eluate from pyruvic oxime position
0–2	180	6	56
2–3	4000	26	344
3–4	5040	10	552
4–5	5040	14	775
5–9	833	18	308
9–21	71	10	88

Five fifth instar larvae each fed with 2 cm^2 leaves coated with 150 μgm pyruvic oxime – $2^{14}C$ (400,000 cpm).

The water extract of the faecal pellets was chromatographed as described in Table 4.

A similar result was obtained when pyruvic oxime – $2^{14}C$ was fed to silkworms (Table 5). The maximum excretion of pyruvic oxime – $2^{14}C$ occurred 4–5 hr after the meal. Chromatography of the aqueous extracts of the faecal pellets again shows that pyruvic oxime is not readily hydrolysed during its passage through the digestive tract of the larvae.

DISCUSSION

Our experiments with pyruvic oxime – $2^{14}C$ clearly show that the metabolism of pyruvic oxime in the silkworm is of an extremely low order and is not comparable with that of pyruvate. Since these are *in vivo* experiments, where the activities of oximase and transoximase are unimpaired by extraction procedures, we conclude that if they are present, as claimed by YAMAFUJI and co-workers, these enzymes are of little importance in the protein synthesis and carbohydrate metabolism of the silkworm. Further, the insects' response to the feeding or injection of pyruvic oxime seems to be that of immediate excretion of a foreign substance. There is no evidence that either oxidation to carbon dioxide or hydrolysis of administered pyruvic oxime occurs since the substance is excreted chemically unchanged. On the other hand, pyruvic acid – $2^{14}C$ is immediately oxidized to $^{14}CO_2$ and the label soon appears in gut and body protein.

VOL. 4 1960

Acknowledgement—We wish to thank Miss F. GREENE and Mrs. L. JAGO for their skilled technical assistance.

REFERENCES

ANGUS T. A. (1954) Use of methyl cellulose in laboratory tests of bacterial pathogens of insects. *Canad. Ent.* **86**, 206.

BERGOLD G. H. (1941) Eine Mikroinjektionsspritze und Mikroburette bis zu 0.1 cmm. *Biol. Zbl.* **61**, 158–162.

BHEEMESWAR B. and FAULKNER P. (1959) Metabolism of oximes in the silkworm *Bombyx Mori* L.—I. Inhibition of transaminases. *J. Ins. Physiol.* **3**, 349–356.

FUKUDA T. (1957) Biochemical studies on the formation of the silkprotein—IV. The conversion of pyruvic acid to alanine in the silkworm larva. *J. Biochem., Tokyo* **44**, 505–510.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L., and RANDALL R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

YAMAFUJI K. (1953) Enzymatic transoximation. *Nature, Lond.* **171**, 745.

YAMAFUJI K. and ETO M. (1953) Chromatographic study of transoximase. *Enzymologia* **16**, 247–255.

YAMAFUJI K., KAWAKAMI T., and SHINOHARA K. (1952) On an enzyme in silkworm which catalyses the transformation of oximes into amino compounds. *Enzymologia* **15**, 199–203.

YAMAFUJI K., OMURA H., and MIURA M. (1953) On the transoximase. *Enzymologia* **16**, 75–80.

YAMAFUJI K., YOSHIHARA F., and WADA H. (1950) Formation of oxime in silkworms after feeding with inorganic nitrogenous salts. *Enzymologia* **14**, 170–176.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 20 *to* 26. *Pergamon Press Ltd., London. Printed in Great Britain*

RESEARCH ON THE MODE OF ACTION OF HALOGEN CONTAINING THIOL ALKYLATING AGENTS ON INSECTS. EFFECT OF IODOACETIC ACID ON CHOLINE ACETYLASE*

M. BOCCACCI, GIUSEPPINA NATALIZI, and S. BETTINI

Department of Parasitology, Istituto Superiore di Sanità, Rome, Italy

(*Received* 19 *March* 1959)

Abstract—The authors have studied the action of iodoacetic acid on choline acetylase of the nervous tissue of *Musca domestica* and *Periplaneta americana*. No inhibition of enzymic activity even at high doses (8 LD_{50}s) of iodoacetic acid has been found.

NACHMANSOHN and MACHADO (1943), in the course of their first studies on choline acetylase, observed that the *in vitro* synthesis of acetylcholine was greatly inhibited by iodoacetic acid and by other substances reacting with thiol groups.

Recently BERMAN (1954) demonstrated that choline acetylase from squid head ganglia is an SH enzyme and that, as such, it is inhibited by iodoacetic acid and by other thiol reagents.

In the course of our research on the mode of action of iodoacetic acid and similar SH reactive analogues (halogen containing thiol alkylating agents) on insects (BETTINI and BOCCACCI, 1952), it was observed that following poisoning with these substances there is a marked decrease of glyceraldehyde-3-phosphate dehydrogenase (BETTINI and BOCCACCI, 1956a) while no evident effect on coenzyme A (BETTINI and BOCCACCI, 1956b) nor on succinic dehydrogenase (BETTINI and BOCCACCI, 1954) could be noted.

Following this line of research, we have studied the effect of iodoacetic acid on insect choline acetylase.

Acetylcholine synthesis by insect nervous tissue has been studied by SMALLMAN (1956) in *Lucilia sericata*, by SMALLMAN and PAL (1957) in the roach, and by FRONTALI (1958) in *Musca domestica*.

METHODS

Biological material. Laboratory-bred strains of *M. domestica* (S-Roma) and of *Periplaneta americana* have been used.

Products. These were obtained commercially as indicated. Acetyl coenzyme A (Bios Laboratories); iodoacetic acid (Light and Co.); coenzyme I and solution of triosephosphates (C. F. Boehringer).

Extracts preparation and enzymic activity determination. Fly heads were dispersed in a Potter–Elvehjem homogenizer at 0°C in a mixture of 2 ml of diethyl

* Presented at the Fourth International Congress of Biochemistry, Vienna, 1–6 September 1958.

ether (peroxide-free) with 5 ml of distilled water containing sodium chloride (11 mg/ml), $MgCl_2.6H_2O$ (3·5 mg/ml), and cysteine hydrochloride (6·0 mg/ml) adjusted to a final pH of 7·2. The homogenate was then shaken at 0°C for 30 min in a wide-mouthed container and the ether allowed to evaporate.

Acetone powders were prepared by the technique described by SMALLMAN (1956). During the preparation of acetone powders extracts the use of diethyl ether was avoided.

For the determination of enzymic activity 2·5 ml of the homogenate were mixed with 2·3 ml of a solution of 0·24 g KCl, 0·112 g NaF, 0·02 g tetraethyl pyrophosphate in 100 ml of 0·02 M phosphate buffer at pH 7·2.

The mixture was then held at 34°C, and 0·1 ml of a 3 per cent choline chloride solution added. A first sample of 1 ml was taken. Immediately after, 0·1 ml of an acetyl coenzyme A (5 mg/ml) solution was added and further samples of 1·0 ml were taken at 1 min intervals for 3 min. The pH of each sample was immediately brought to 4·5 by the addition of 0·1 N HCl and the sample inactivated by heating in a boiling water bath for 1 min, centrifuged and adjusted to pH 7·0. The acetylcholine formed was determined by the method of APRISON and NATHAN (1957).

VOL. 4 1960

The quantity (μg) of acetylcholine synthesized during the first minute of reaction by 1 mg of acetone powder or by 6 mg of fresh tissue was taken as an index of enzymic activity (about 1 g of acetone powder is obtained from 6 g of fresh tissue).

Preparation of the purified enzyme. The method of BERMAN, WILSON, and NACHMANSOHN (1953) was followed for the preparation of purified choline acetylase. An enzymic preparation with a five-fold increase in specific activity was thus obtained.

In the *in vitro* inhibition tests with iodoacetic acid the determination of enzymic activity was made by BERMAN'S (1954) method.

Determination of glyceraldehyde-3-phosphate dehydrogenase activity. A method based on the spectrophotometric determination of reduced coenzyme I, following the indications reported by CHEFURKA (1954), was used.

The extracts were prepared with a medium containing cysteine.

Iodine determinations. Iodoacetate absorbed by flies during exposure was estimated through iodine determination. For this purpose a method for iodine determination due partly to STEVEN (1936) and partly to MARIANI-MARELLI and MARIANI (1955) was used: to 25–50 flies in a 100 ml Pyrex flask, the following solutions were added: 2 ml of a 0·5 per cent solution of ceric sulphate in 3 per cent sulphuric acid, 2·5 ml of an aqueous saturated solution of chromic acid, and 20 ml of concentrated sulphuric acid. The temperature was raised to 220°C for 5 min. After cooling and adding 20 ml of saturated oxalic acid, the solution was steam distilled. About 50 ml of distillate was collected in a flask containing 1 ml of N NaOH. After concentration to about 10 ml, the distillate was transferred to a small separating funnel. The following were then added: 2·5 ml of carbon tetrachloride, 0·5 ml of 7 per cent sodium nitrite in water and 1 ml of dilute sulphuric acid. The mixture was shaken for about 1 min and allowed to separate. The carbon

tetrachloride was collected and cleared over anhydrous sodium sulphate. Colour measurements were made within 5 min at 516 mμ.

Treatment of insects with iodoacetic acid. The roaches were injected with a sodium iodoacetate solution at pH 7·0 by inserting the microsyringe needle between the third and fourth tergite laterally to the median line of the abdomen. The flies were placed in contact with filter paper treated with an olive oil solution of iodoacetic acid. LD_{50} values were estimated as already described (BETTINI and BOCCACCI, 1956a).

RESULTS

Choline acetylase activity in fly and roach heads. Table 1 shows the normal values of choline acetylase activity found in fly and roach heads. It can be observed that the enzymic activity of fresh fly tissue homogenates is about equal to that of acetone powder extracts due to the use of ethyl ether during the homogenizing procedures (HEBB and SMALLMAN, 1956).

TABLE 1—CHOLINE ACETYLASE ACTIVITY FROM HEADS OF *M. domestica* AND *P. americana*

Insect species	Acetone dried powder μg Ach/mg powder/1 min	Fresh tissue μg Ach/6 mg tissue/1 min*
M. domestica	1·60–1·80	1·60–2·20
P. americana	—	0·30–0·50
L. sericata (Smallman)	1·30–1·70	—

* The enzymic activity is referred to 6 mg of fresh tissue for direct comparison with acetone dried powder (1 g of acetone powder is obtained from 6 g of fresh heads).

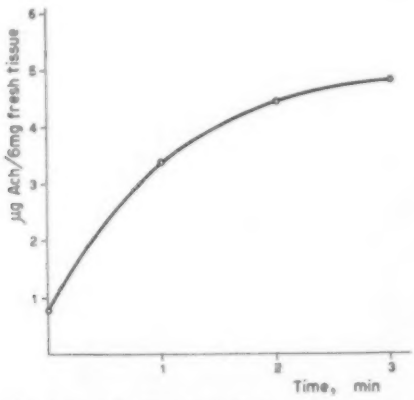


FIG. 1. Synthesis of acetylcholine from fly head homogenate, against time.

The choline acetylase activity values found by us in *M. domestica* are in agreement with those reported by SMALLMAN (1956) for *Lucilia sericata*.

In Fig. 1 is shown the rate of acetylcholine synthesis from a fly-head homogenate.

Effect of age on choline acetylase activity of house flies. Before studying the effect of iodoacetic acid, the choline acetylase activity from heads of flies at different ages was determined in order to employ individuals at the age of highest enzymic activity.

HEBB (1956) found an increased choline acetylase activity in the nervous tissue of rabbit and guinea-pig during the late period of embryo development and the first days after birth. Increases in various substances have also been noted

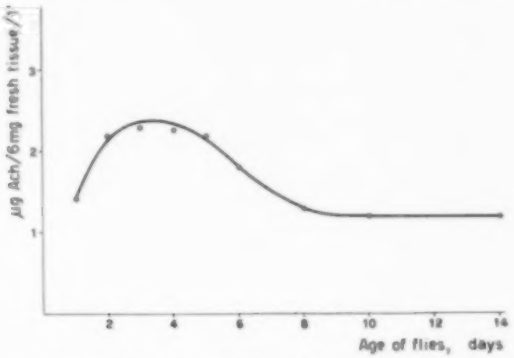


FIG. 2. Choline acetylase activity from heads of *M. domestica* at different ages. (The value of each point is the average of 2 determinations on groups of 300 flies.)

during the first days of adult life of some insects, e.g. in cytochrome-*c* (LEVENBOOK and WILLIAMS, 1956), in coenzyme A (BOCCACCI and BETTINI, 1956) and in cytochrome oxidase activity (SACKTOR, 1951) parallel with an increase of tolerance for insecticides (KERR, 1954).

TABLE 2—AMOUNTS OF IODOACETIC ACID TAKEN UP BY MALES AND FEMALES OF *M. domestica* KEPT IN CONTACT WITH DIFFERENT CONCENTRATIONS OF THE ACID

Iodoacetic acid		
g/m²	µg/fly	
	Males	Females
0·35 (1 LD_{50} males)	0·81 ± 0·04	—
0·70	1·38 ± 0·06	—
0·85 (1 LD_{50} females)	—	2·45 ± 0·11
1·50	2·23 ± 0·10	—
1·75	—	3·30 ± 0·16
3·00	6·00 ± 0·30	—
3·50	—	7·10 ± 0·35
6·00	—	12·00 ± 0·52

The variation of choline acetylase activity with age in *M. domestica* is shown in Fig. 2. Enzymic activity increases greatly until the second day of age, then it remains at constant level until the fifth day when it starts to decrease.

Flies 3–5 days old were employed in the following experiments.

Iodoacetic acid taken up by flies. Results based on the iodine determinations together with the estimated LD_{50} values are summarized in Table 2.

Effects of iodoacetic acid in vivo *on choline acetylase.* As Table 3 shows, treating insects with high doses of iodoacetic acid, corresponding to 10 LD_{50}s, does not influence the choline acetylase activity from fly and roach heads.

TABLE 3—CHOLINE ACETYLASE ACTIVITY FROM HEADS OF *M. domestica* AND OF *P. americana* TREATED WITH IODOACETIC ACID

Insect species	Iodoacetic acid administered μg/individual		Inhibition of enzymic activity %
	Males	Females	
M. domestica	0·81 (1 LD_{50})	2·45 (1 LD_{50})	0
	1·30	3·30	0
	2·23	7·10	0
	6·00	11·00	0
P. americana		180 (2 LD_{50})	0
		360 (4 LD_{50})	0
		1800 (10 LD_{50})	0

TABLE 4—*In vivo* EFFECT OF IODOACETIC ACID ON GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITY OF HOUSEFLY HEADS AND OF ROACH GANGLIONIC CHAIN

Insect species	Iodoacetic acid μg/individual (females)	Inhibition of enzymic activity %
M. domestica (heads)	2·0 (LD_{20})	15
	3·0 (LD_{85})	75
	4·0 (LD_{100})	100
P. americana (ganglionic chain)	100 (LD_{50})	40
	300 (3 LD_{50})	70

The determinations of enzymic activity were carried out 1 hr after treatment with iodoacetic acid.

Effects of iodoacetic acid on glyceraldehyde-3-phosphate dehydrogenase in vivo. The lack of choline acetylase inhibition *in vivo* might have been due to impermeability of the nerve sheet to iodoacetic acid. To test this we determined the glyceraldehyde-3-phosphate dehydrogenase activity in nervous tissue (ganglionic chain of roaches and heads of flies) of groups of treated individuals as an indication

of iodoacetate penetration. We have chosen the inhibition of glyceraldehyde-3-phosphate dehydrogenase as an index of iodoacetic acid penetration in nervous tissue since in our preceding work (BETTINI and BOCCACCI, 1956a) it was found that the insect enzyme is very sensitive to the action of iodoacetic acid as in the case of vertebrates.

As can be seen from Table 4, even at the lowest doses of iodoacetic acid, there is a marked inhibition of glyceraldehyde-3-phosphate dehydrogenase from the heads of flies as well as from the ganglionic chain of roaches. This clearly indicates that iodoacetic acid penetrates into the nervous tissue.

TABLE 5—*In vitro* EFFECT OF IODOACETIC ACID ON PURIFIED CHOLINE ACETYLASE OF HEADS OF *M. domestica*

Iodoacetic acid (molar concentration)	Inhibition of enzymic activity %
10^{-4}	0
10^{-3}	0
10^{-2}	0
10^{-1}	30

Effects of iodoacetic acid in vitro *on choline acetylase.* The lack of enzyme inhibition *in vivo* could have been explained by low reactivity of choline acetylase of insects with iodoacetic acid. We have, therefore, tested the *in vitro* inhibition on a purified enzyme. As can be observed from Table 5, iodoacetic acid shows a mild effect only at very high concentrations.

CONCLUSION

The above results strongly suggest that choline acetylase from insect nervous tissue behaves differently toward iodoacetic acid than squid's head ganglia choline acetylase (BERMAN, 1954).

It has been shown that iodoacetic acid penetrates the insect nerve sheet which is notoriously impermeable to several ionized substances (O'BRIEN, 1958). This finding should be particularly interesting for the understanding of the mode of action of iodoacetic acid poisoning.

Acknowledgements—Thanks are due to Dr. NORA FRONTALI for useful suggestions in the course of this work and to Mr. G. PIERDOMINICI for technical assistance.

REFERENCES

APRISON M. H. and NATHAN P. (1957) Determination of acetylcholine in small samples of fresh brain tissue. *Arch. Biochem. Biophys.* **66**, 388.

BERMAN R. R. (1954) Sulfydril groups of choline acetylase. *Biochim. biophys. Acta* **14**, 442.

BERMAN R., WILSON J. B., and NACHMANSOHN D. (1953) Choline acetylase specificity in relation to biological function. *Biochim. biophys. Acta* **12**, 315.

VOL. 4 1960

Bettini S. and Boccacci M. (1952) Sostanze alogenate alchilanti come insetticidi di contatto per le mosche resistenti. *R. C. Ist. sup. Sanità* **15**, 609.

Bettini S. and Boccacci M. (1954) Localizzazione e distribuzione della succinodeidrogenasi nel muscolo di insetti. *R. C. Ist. sup. Sanità* **17**, 188.

Bettini S. and Boccacci M. (1956a) Toxic action of iodo- and chloroacetic acid on insects. Inhibition of triosephosphate dehydrogenase. *Sel. Sci. Pap. Ist. sup. Sanità* **1**, 155.

Bettini S. and Boccacci M. (1956b) Effects of iodoacetic acid on the coenzyme A content of the muscle of the American cockroach. *J. econ. Ent.* **49**, 554.

Boccacci M. and Bettini S. (1956) Coenzyme A content in the American cockroach (*Periplaneta americana* L.) and the housefly (*Musca domestica*). *Experientia* **12**, 432.

Chefurka W. (1954) Oxidative metabolism of carbohydrates in insects. I-Glycolysis in the housefly *Musca domestica* L. *Enzymologia* **17**, 73.

Frontali N. (1958) Acetylcholine synthesis in the housefly head. *J. Ins. Physiol.* **1**, 319.

Hebb C. (1956) Choline acetylase in the developing nervous system of the rabbit and guinea pig. *J. Physiol.* **133**, 566.

Hebb C. and Smallman B. N. (1956) Intracellular distribution of choline acetylase. *J. Physiol.* **134**, 385.

Kerr R. W. (1954) Variation with age in the susceptibility to DDT and the respiration rate of male and female *Drosophila melanogaster* Mg. *Bull. ent. Res.* **45**, 323.

Levenbook L. and Williams C. M. (1956) Mitochondria in the flight muscles of insects —III. Mitochondrial cytochrome in relation to the aging and wing beat frequency of flies. *J. gen. Physiol.* **39**, 497.

Mariani-Marelli O. and Mariani A. (1955) La determinazione fotometrica dello iodio nelle sostanze organiche. *R. C. Ist. sup. Sanità* **18**, 225.

Nachmansohn D. and Machado A. L. (1943) The formation of acetylcholine. A new enzyme: "choline acetylase". *J. Neurophysiol.* **6**, 397.

O'Brien R. D. (1958) An ion-impermeable barrier in the roach. *Proc. 10th int. Congr. Ent.* **2**, 377.

Sacktor B. (1951) A comparison of cytochrome oxidase activity of two strains of house flies. *J. econ. Ent.* **43**, 832.

Smallman B. N. (1956) Mechanisms of acetylcholine synthesis in the blowfly. *J. Physiol.* **132**, 343.

Smallman B. N. and Pal R. (1957) The activity and intra-cellular distribution of choline acetylase in insect nervous tissue. *Bull. ent. Soc. Amer.* **3**, 25.

Stevens C. D. (1936) Determination of iodine in biological materials. A simplified technique. *J. Lab. clin. Med.* **22**, 1074.

J. Ins. Physiol., 1960, Vol. 4, pp. 27 to 37. Pergamon Press Ltd., London. Printed in Great Britain

LOCOMOTOR ACTIVITY OF THE BLOWFLY AS A FUNCTION OF FEEDING AND STARVATION*

L. BARTON BROWNE† and D. R. EVANS

The Johns Hopkins University, Baltimore 18, Md.

(*Received* 1 *April* 1959)

Abstract—The effect of feeding and starvation on locomotor activity in *Phormia regina* Meigen was studied. It was found that flies fed glucose, fucose, or mannose were much less active than were flies that had been starved for 24 hr. Immediately after feeding, flies were less active than at any other time, but activity increased progressively thereafter. Metabolic state and blood sugar concentration were shown not to be determinants of activity; nor was the mechanism involved in the control of activity identical with that by which feeding and starvation control taste threshold. Evidence was given that locomotor activity is some function of crop volume and hence, perhaps, of the rate of crop emptying. Injections greatly decreased activity, partly by means of a wound factor type of action.

VOL. 4 1960

INTRODUCTION

THE relationship between locomotor activity and food deprivation has been little studied in insects. EDNEY (1937) provided the first quantitative data when he showed starvation for a few hours caused a marked increase in the 'spontaneous' activity of the locust *Locusta migratoria migratorioides*. Further evidence is given in later papers by ELLIS (1951) and CHAPMAN (1954). HOYLE (1954) showed that the potassium concentration in the blood was higher in grass-fed than in starved locusts and gave convincing evidence that the higher potassium caused a reduction in the mechanical responses of the muscles to nerve stimulation. High potassium is known to reduce end-plate potentials, muscle membrane resting potentials, and active membrane response in *Locusta* (HOYLE 1954, 1955). ELLIS and HOYLE (1954) demonstrated that high potassium diets reduced marching activity in *Locusta*, whereas diets containing little or no potassium were ineffective. These authors concluded that the observed effects were due to differences in muscular efficiency.

It is obvious that the explanation given by ELLIS and HOYLE can be invoked to account for the relationship between locomotor activity and feeding only in insects which have access to foods rich in potassium. The blowfly *Phormia regina* is an insect which probably feeds mainly on sugars and proteins and hence would not normally take in significant amounts of potassium. Threshold of response to sugars by *P. regina* has been shown to increase after feeding; and much is known about the mechanism of this interaction (DETHIER *et al.*, 1956; EVANS and DETHIER,

* This investigation was supported by research grant B-1282 from the National Institute of Neurological Diseases and Blindness, Public Health Service.

† Fulbright Research Scholar. On leave from C.S.I.R.O., Canberra, Australia.

1957; DETHIER and BODENSTEIN, 1958; EVANS and BROWNE, in preparation). The experiments described in this paper were designed to determine whether or not the locomotor activity of *Phormia* is reduced by the intake of sugar and, if so, to determine whether or not the mechanism by which feeding affects activity is similar to that by which it affects the threshold of response to sugars.

METHODS

(a) *Standardization of flies*

(i) *Starved flies.* Since a high degree of standardization was found to be desirable, the flies were given special treatment from the time of their emergence. The flies emerged in 1 quart Mason jars and, during their first day, filter papers moistened with 0·1 M sucrose were supplied. At noon on their second day the flies were counted under carbon dioxide and batches of 100 placed in clean jars containing fresh filter paper moistened with 0·1 M sucrose. At noon on the third day the filter papers were again replaced with fresh ones which were removed after 30 min; then the flies were transferred without anaesthesia to clean jars containing filter papers moistened only with distilled water. At noon on their fourth day the flies were ready for the measurement of their activity.

(ii) *Fed flies.* These were treated in the same way as starved flies until noon on their fourth day, at which time filter papers moistened with appropriate sugar solutions were placed in the jars for 30 min. This time was sufficient for the flies to feed to repletion.

(b) *The measurement of locomotor activity*

The apparatus used for measuring the locomotor activity of the flies is shown in Fig. 1. It consisted of four cardboard boxes, numbered 1–4. Each box was connected with the next by a stemless glass funnel as shown in Fig. 1. Both the

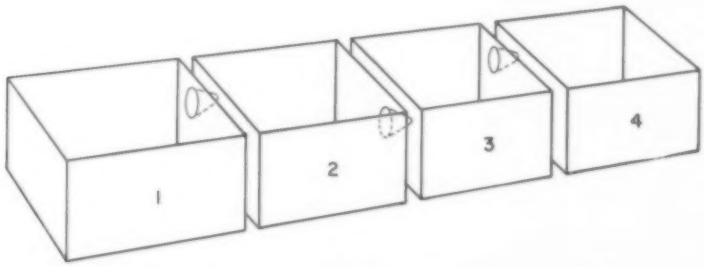


FIG. 1. The apparatus used for measuring locomotor activity.

large and small ends of the funnels were flush with the surface of the boxes in which they were set. The "Plexiglas" top of each box was removable. Flies (about 100 unless otherwise stated) were introduced into box 1 through an opening in its side. In order that they might recover from the handling and adapt to the lighting conditions to be used during the activity determination, the flies were held

in box 1 for one hour by a stopper in the funnel between boxes 1 and 2. At the end of an hour the funnel was opened and the numbers of flies in boxes 2, 3, and 4 were counted at regular intervals thereafter. Funnels rather than holes were provided between the boxes in order to make the system biased in one direction; the chance of flies finding the larger end was greater than that of finding the smaller. In most experiments the system was made more effectively biased by the use of a light source situated beyond box 4. The bias increased the sensitivity of the apparatus.

From the numbers of flies in boxes 2, 3, and 4 at the beginning and end of a given time period, it was possible to determine the net number of 'funnel passages' that had occurred. A measure of locomotor activity for the time interval was obtained by expressing the number of 'funnel passages' occurring as a percentage of the number of 'funnel passages' which would occur if all the flies moved from the boxes they were in at the beginning of the period into box 4. Time periods of 5, 15, and 60 min were used depending on the rate of movement; but, in all cases, the final rate has been converted to per cent/15 min. All means and standard errors were derived from the rates for a number of time intervals obtained from at least two separate batches of flies.

VOL. 4 1960

Most of the experiments were performed in a room controlled at a temperature of either 27 or 30°C. A few, however, were done under uncontrolled temperature conditions. In the latter, paired trials were carried out in two identical sets of boxes and the results obtained were compared only with one another. All the experiments were carried out in the early afternoon except those dealing with activity as a function of time after feeding (which were begun at about 9 a.m.).

RESULTS

1. *Preliminary experiments*

(a) *Testing the equivalence of the two sets of boxes.* An experiment was carried out to ascertain whether the two sets of boxes used were equivalent in their performance. Identically treated flies were concurrently tested against one another. The results obtained from the two sets of boxes were very similar; the rates being $9{\cdot}6 \pm 1{\cdot}2$ and $10{\cdot}1 \pm 0{\cdot}9$ per cent/15 min.

(b) *Males vs. females.* Two trials were performed in which males were tested against females. In one the flies were starved and in the other they were fed 2 M glucose. The rates for males and females in each test were almost identical. When the flies were starved the rate for males was $13{\cdot}0 \pm 0{\cdot}3$ per cent/15 min and that for females was $12{\cdot}8 \pm 0{\cdot}3$ per cent/15 min ($P > 0{\cdot}5$). For fed flies the rates were, for males, $3{\cdot}6 \pm 0{\cdot}8$ per cent/15 min and, for females, $3{\cdot}5 \pm 0{\cdot}8$ per cent/15 min ($P > 0{\cdot}5$). The results showed that in future experiments it was not necessary to have equal numbers of males and females in samples for testing.

(c) *Effect of water deprivation.* A comparison was made between starved flies which had been in the presence of filter paper moistened with distilled water for 24 hr and starved flies given only dry filter paper. Again the results were almost identical; the rates being, for moist, $10{\cdot}6 \pm 1{\cdot}0$ per cent/15 min and, for dry,

$10{\cdot}4 \pm 1{\cdot}5$ per cent/15 min ($P > 0{\cdot}5$). Since water had no effect, it was decided to supply the flies routinely with moist filter paper during the period of starvation, in order to obtain better survival.

2. *Determination of the rates of funnel passages under different lighting conditions*

(a) *Strongly directional light.* The experiments described in this section were done in a darkroom controlled at 30°C. The only source of illumination was a fluorescent light placed slightly above and beyond box 4. With this lighting arrangement the flies were under a very strongly directional light stimulus, but the overall illumination was low. The funnel passage rate of four groups of flies was determined:

(i) Starved flies.
(ii) Flies fed on 2 M glucose 1 hr before the experiment.
(iii) Flies fed on 1 M fucose 1 hr before the experiment.
(iv) Flies fed on a mixture containing 0·5 M fucose and 0·05 M sucrose 29 hr before the experiment.

The results obtained are given in Table 1. Ingestion of glucose and fucose decreased activity, the latter more so. The flies of Group (iv) were equivalent in activity to starved flies.

TABLE 1—THE FUNNEL PASSAGE RATES UNDER STRONGLY DIRECTED LIGHT FOR FLIES GIVEN DIFFERENT FEEDING TREATMENTS

Treatment	No. of time intervals during which counts were made	No. of separate batches of flies	Activity f.p. per cent/15 min	
			Mean	S.E.
Starved	11	4	15·6*	±0·56
Fed 2 M glucose —tested 1 hr later	21	7	3·9	±0·53
Fed 1 M fucose —tested 1 hr later	8	2	0·9	±0·29
Fed 0·5 M fucose and 0·05 M sucrose; tested ca. 29 hr later	4	2	14·0*	±3·6

* Not significantly different at 0·5 level. All other means are significantly different from one another at 0·01 or 0·001 level.

(b) *Darkness.* A series of experiments was done at 30°C in which the flies were in complete darkness except for a short period at the end of each time interval when counts were made. These periods of illumination caused increased activity in the flies, but in no case was a fly seen to make a funnel passage while the light was on; and the activity of the flies decreased quickly upon return to darkness. It was assumed, therefore, that these short periods of light did not have a

significant effect upon the results. In order to increase the number of funnel passages to reasonably large values it was found necessary to use 200 flies and a time interval of 1 hr. The rates were, for starved flies, $1{\cdot}3 \pm 0{\cdot}5$ per cent/15 min and, for fed, $0{\cdot}2 \pm 0{\cdot}1$ per cent/15 min. These rates were significantly different ($P < 0{\cdot}05$).

(c) *Box* 1 *blackened.* An experiment was performed in which the arrangement was similar to that described in Section 2(a) except that box 1 was blackened so that the only light within it was from the glass funnel. Under this arrangement the flies in box 1 were under a very low illumination but had a discrete light source as a directive stimulus. In these trials only the flies passing from box 1 to box 2 were counted. Starved flies and flies fed 2 M glucose were tested. The rate for starved flies was $168 \pm 4{\cdot}5$ per cent/15 min and that for fed flies was $34{\cdot}5 \pm 5.4$ per cent/15 min. The means are significantly different ($P < 0{\cdot}001$). In this experiment the high rates obtained necessitated the use of a 5 min time interval.

(d) *Weakly directional light.* In a series of experiments, done prior to the availability of the constant temperature room, the lighting conditions were again different. The room in which the experiments were done received most of its light through a window beyond box 4 but because of other sources the result was a less directed light stimulus than those described in Sections (a) and (c). The overall level of illumination was, however, much higher. As the conditions were not standard the tests were always paired. In each trial a batch of flies fed on 2 M glucose or 1 M fucose was run concurrently with a starved batch in the other set of boxes. The mean starved to glucose-fed ratio was $4{\cdot}1 \pm 0{\cdot}3$ and the mean starved to fucose-fed ratio was $3{\cdot}9 \pm 0{\cdot}2$.

VOL. 4 1960

3. *Change in activity with time after feeding*

A series of experiments was done in which the rates of funnel passages were determined at intervals of 1 hr, 3 hr, 6 hr, and 24 hr after ingestion of glucose and mannose. The lighting conditions used were those described in Section 2(a) in which the light was strongly biased and the temperature was 27°C. In periods between the tests the light beyond box 4 was kept off and one before box 1 left on. This light held the flies in box 1 and tended to cause those which had already left box 1 to return to it. Not all of these always returned to box 1 because of the bias of the funnels. For this reason, approximately 150 flies were used in each trial to ensure that even after 24 hr there was still a large number of flies (ca. 100) which had not reached box 4. The results are shown in Fig. 2. It is clear that both the glucose- and mannose-fed flies showed an increase in activity with time. By the end of 24 hr the rate of funnel passages for flies fed on glucose was about the same as that for starved flies. Rates for flies fed on mannose showed a less rapid increase with time, their rate of funnel passages at the end of 24 hr had only increased to about that of glucose-fed flies 6 hr after feeding. In most cases readings were not obtained between 6 and 24 hr because it was found that the flies showed a diurnal rhythm of activity in constant light and that only between 8 a.m. and 5 p.m. was

the activity of starved flies constant. At night the activity fell as low as half of the expected value. To illustrate this, one such point is included in the curve for glucose-fed flies.

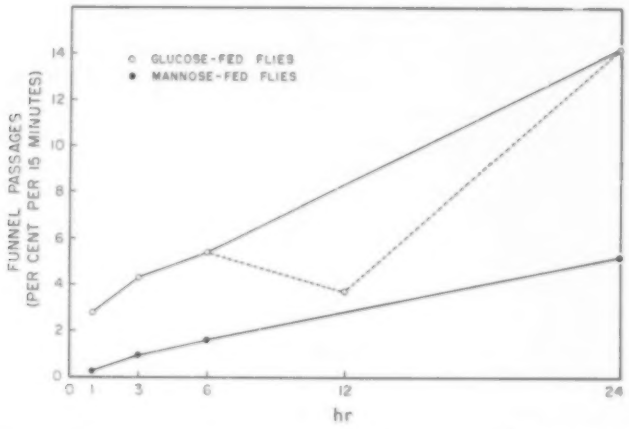


FIG. 2. The change in locomotor activity with time after feeding to repletion on 2 M glucose and 2 M mannose.

4. *The rate of funnel passages after injection*

A series of experiments was done in which the activities of starved flies which received injections of sugar solution or water were determined. The injections were given in the abdomen through an intersegmental membrane by means of a fine glass needle which was cemented to the needle of a tuberculin syringe. A volume of 2·86 μl was introduced into each fly, using a microinjection apparatus (BUCK, 1949) calibrated as suggested by EVANS and DETHIER (1957). The rates of funnel passages of flies which received injections of 2 M glucose, of flies which received injections of distilled water, and of flies which merely had a dry needle inserted into their abdomens, were determined. In all these tests the first box was blackened and the temperature was 30°C as described in Section 2(c). The tests were run 1 hr after injection. The mean rate of funnel passages for flies which received glucose injections was $6{\cdot}0 \pm 1{\cdot}5$ per cent/15 min, whereas that for those which received water injections was $16{\cdot}8 \pm 3{\cdot}3$ per cent/15 min. The 'stabbed' flies gave a rate of 153 per cent which was very little less than that given by normal starved flies. It is clear that the injections of sugar and water made the flies much less active than normal flies. The flies which received glucose injections had a significantly lower rate than those which received water injections ($P < 0{\cdot}01$), but the difference between the rates for these two groups was not of the same order as the difference between the rates for either of these groups and that for the stabbed flies.

Larger needles (e.g. 27 gauge hypodermic needles) inserted without injection into various regions of the abdomen and thorax greatly decreased activity, an effect

of the wounding alone. This result contrasts with the lack of effect of several extensive operations and of injections on other complex behavioural responses of the blowfly (taste thresholds and feeding: DETHIER *et al.*, 1956; flight and taste thresholds: HUDSON, 1958). Thus wounding of various hypodermal regions rather specifically inhibits locomotor activity.

DISCUSSION

Funnel passage rates as a measure of activity

Before it is possible to discuss the significance of the results obtained it is necessary to know the relation between the funnel passage rates obtained and 'spontaneous' locomotor activity. The concept of true spontaneous activity is an abstraction in that an organism cannot be divorced entirely from its environment from which it always receives some sensory stimulation. For practical purposes, however, the term 'spontaneous' activity has been used to designate the activity of organisms when all obvious components of the environment are kept constant and uniform within the area in which the organism is allowed to move. In the experiments described in this paper only the measurement of activity in complete darkness approaches this situation. Under these conditions the ratio of the rate of starved flies to the rate of glucose-fed flies was 6·5 to 1, but because of the low funnel passage rate the results are not as reliable as those obtained under other conditions.

VOL. 4 1960

No attempt was made to perform experiments under uniform light because of the technical difficulties in obtaining such an arrangement. Experiments were, therefore, done under a number of conditions of directed light. The ratios obtained for the rates of starved flies to the rates of glucose-fed flies under the various conditions were, for strongly directed light, 4·0 to 1; for box 1 blackened, 4·9 to 1; and for slightly directed light, 4·1 to 1. These ratios are very close to one another and agree fairly well with those obtained for similarly-treated flies in complete darkness. Thus, it seems likely that feeding has no marked effect on the light reactions of *Phormia*, such as a clear-cut reversal of light response as described by WELLINGTON (1948) for *Choristoneura fumiferana*. It is justifiable, therefore, to use the relative funnel passage rates for flies given different feeding treatments under conditions of directed light as a measure of their relative 'spontaneous' activities.

The mechanism by which feeding affects locomotor activity

When efforts are made to disturb fed flies by making movements in their vicinity it was found that, although they were less easily disturbed than starved flies, they were capable of both flying and walking. Furthermore, fucose-fed flies, which ingest a smaller volume of sugar solution than glucose-fed flies (EVANS and DETHIER, 1957), are less active. It must be concluded, therefore, that the reduction in activity after feeding is not due merely to an inability to move caused by increase in weight. Since fucose, a sugar which is not utilized by *Phormia*, was even more

effective than the readily utilized sugar, glucose, in rendering flies inactive, it is clear that, like taste threshold, activity is not controlled by the metabolic state of the fly.

Flies starved for 29 hr after ingestion of 0·5 M fucose and 0·05 M sucrose had virtually empty crops and low taste thresholds (< 0·5 M to glucose) but had a blood sugar, probably mainly fucose (EVANS and DETHIER, 1957), concentration of 28·8 g/l. (expressed as fucose, as determined by EVANS and DETHIER, 1957) which is very much higher than that of normal starved flies (EVANS and DETHIER, 1957). Despite their high blood sugar these flies were as active as starved ones (Table 1). Since fucose is as effective in reducing activity as mannose or glucose, it seems safe to assume that it affects activity by the same mechanism as these metabolizable sugars. It is probable, therefore, that blood sugar concentration does not affect activity.

In Fig. 3 can be seen both activity and glucose threshold plotted against crop volume for both glucose- and mannose-fed flies. The figures for activity are those given previously in Fig. 2. The crop volumes against which activity was plotted

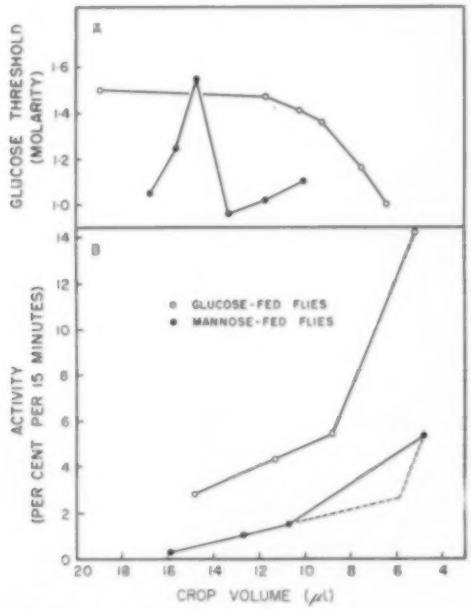


FIG. 3. A. The relation between crop volume and glucose taste threshold. B. The relation between crop volume and locomotor activity.

were obtained by weighing the crops of flies kept in Mason jars under the same conditions of temperature and light as those used for activity measurements. Since the concentration of sugar in the crop is the same as that of the sugar solution

which the fly has imbibed (EVANS and DETHIER, 1957) it is valid to obtain the crop volumes from the crop weights and the specific gravity of the solution on which the flies fed. The data for threshold after glucose and mannose feeding were obtained from EVANS and DETHIER (1957) and were plotted against data derived from the same source. These were chosen because they had been obtained under the same conditions as had the threshold data, and because in each case the flies were not free but were mounted on wax sticks.

That the relationship between crop volume and threshold for flies fed on glucose is very different from that for flies fed on mannose is shown in Fig. 3A. On the other hand Fig. 3B shows that the relationships between crop volume and activity are more similar for flies fed on the two sugars. Hence the relationships between threshold and activity for flies fed on the two sugars are very different. The shape of the curve relating threshold to time (and hence crop volume) after mannose ingestion was found to be extremely reproducible (EVANS and DETHIER, 1957; EVANS and BROWNE, in preparation) and agrees generally in shape with that obtained for fucose-fed flies (EVANS and DETHIER, 1957). It seems certain, therefore, that the curve for mannose-fed flies (Fig. 3A) shows the true relationship between threshold-time after feeding. It must be concluded, then, that the mechanism by which feeding controls activity is not identical with that by which feeding controls taste threshold.

VOL. 4 1960

Since the mechanism whereby feeding controls activity involves neither the metabolic state of the fly nor the blood sugar, and it is not identical with threshold, it is necessary to discuss the remaining possibilities and to assess the evidence for and against each. It is shown in Fig. 3B that the slope of the activity versus crop volume curve for mannose is approximately parallel with the curve for glucose up until the crop volume has fallen to about 10 μl. This indicates that activity is probably a function of crop volume. The control of activity by crop volume could be brought about in a number of ways. For example, the controlling factor may be (i) the distension of the crop itself or of the body wall, or (ii) the rate of crop emptying, which is known to decrease as the crop volume diminishes (EVANS and DETHIER, 1957). The rate of crop emptying would (a) determine the frequency of stimulation of any receptors which exist in the gut and (b) determine the rate of passage of sugar solutions into the blood.

No direct evidence was obtained for or against the role of crop or body-wall distension, but WELLINGTON (1948) has shown that in the spruce budworm *Choristoneura fumiferana* the light reactions are reversed when the gut is distended and that the mechanism is the exertion of pressure by the gut on the fifth abdominal ganglion. Also, WIGGLESWORTH (1934) has shown that distension of the abdomen in *Rhodnius prolixus* plays an important part in moulting by providing the stimulus which caused secretion of the moulting hormone. Hence, it is clear that distension of the crop or the body wall can play important roles in the physiological processes of insects. It is possible, therefore, that such mechanisms could operate in the control of locomotor activity of *Phormia*. The fact that fucose-feeding causes a greater reduction in activity than does glucose-feeding is, however, indirect

evidence against distension as a controlling factor, since fucose intake is less than glucose.

This greater effect of fucose ingestion on activity may also be interpreted as evidence that internal receptors may be involved. It is known that 1 M fucose can cause greater threshold rise than 2 M glucose (Evans and Dethier, 1957) and this suggests that the former is possibly more stimulating to internal receptors. There are, however, other possible explanations for this phenomenon, since the total amount of emptying from the crop in the time period from 3–6 hr after feeding is higher for fucose-fed than for glucose-fed flies, and it is after 6 hr that the threshold for fucose-fed flies reaches its peak value.

There is some evidence that activity may be controlled by the degree of blood dilution, which would be a function of the rate of crop emptying and of crop volume. The experiment in which the flies were injected with a sugar solution or distilled water showed clearly that the sudden introduction of 2·86 μl of either into their blood caused a marked reduction in activity. The difference between the activities of flies which receive sugar and water injections does not necessarily conflict with the conclusion drawn above that the blood-sugar concentration is of no significance. The sugar solution would probably, in effect, have had a greater diluting effect on the blood constituents than the distilled water, due to osmotic withdrawal of water from the tissues into the blood. Since it is known that some insects are able to regulate the concentration of the mineral constituents of their blood (Asperen and Esch, 1956; Chapman, 1958), perhaps the rate at which the blood is being diluted is important. When crop emptying is slow, the amount of sugar solution being absorbed through the midgut is very small during a given time interval and, in fact, may be so slow that regulation may be able to keep pace with the rate of entry of the solution into the blood. It is conceivable that the dilution of all the ionic constituents of the blood could impair the efficiency of nervous or neuromuscular transmission or of muscular contraction. Another possible mechanism is that the rate of regulation of the concentrations of some ions may be more rapid than that of others, e.g. magnesium concentration may increase more rapidly after dilution than does calcium concentration. Robertson (1957) has postulated an ionic control of activity in decapod Crustacea; he suggests that the more sluggish species are in a state of partial magnesium narcosis. But it is clear that, whatever the mechanism, the activity is affected by the ingestion of foods containing no potassium. It is very unlikely that the potassium concentration of the blood is increased by feeding on fucose or on the metabolizable sugars; and, therefore, it seems that some explanation other than that given by Ellis and Hoyle (1954) is needed to explain the reduction of activity after feeding and injection of *Phormia*.

REFERENCES

Asperen K. van and Esch I. van (1956) The chemical composition of the haemolymph in *Periplaneta americana* with special reference to the mineral constituents. *Arch. néerl. Zool.* **11**, 342–360.

Buck J. B. (1949) A versatile microinjection and micropipetting syringe. *Rev. sci. Instrum.* **20**, 676–677.

CHAPMAN R. F. (1954) Responses of *Locusta migratoria migratorioides* (R. and F.) to light in the laboratory. *Brit. J. anim. Behav.* **2**, 146–152.

CHAPMAN R. F. (1958) A field study of the potassium concentration in the blood of the Red Locust *Nomadacris septemfasciata* (Serv.) in relation to activity. *Anim. Behav.* **6**, 60–67.

DETHIER V. G. and BODENSTEIN D. (1958) Hunger in the blowfly. *Z. Tierpsychol.* **15**, 129–140.

DETHIER V. G., EVANS D. R., and RHOADES M. V. (1956) Some factors controlling the ingestion of carbohydrates by the blowfly. *Biol. Bull., Woods Hole* **111**, 204–222.

EDNEY E. B. (1937) A study of spontaneous locomotor activity in *Locusta migratoria migratorioides* (R. and F.) by the actograph method. *Bull. ent. Res.* **28**, 243–278.

ELLIS P. E. (1951) The marching behaviour of hoppers of the African migratory locust (*Locusta migratoria migratorioides* R. and F.) in the laboratory. *Anti-Locust Bull.* **7**.

ELLIS P. E. and HOYLE G. (1954) A physiological interpretation of the marching of hoppers of the African migratory locust (*Locusta migratoria migratorioides* R. and F.). *J. exp. Biol.* **31**, 271–279.

EVANS D. R. and DETHIER V. G. (1957) The regulation of taste threshold for sugars in the blowfly. *J. Ins. Physiol.* **1**, 3–17.

HOYLE G. (1954) Changes in the blood potassium concentration of the African migratory locust (*Locusta migratoria migratorioides* R. and F.) during food deprivation, and the effect on neuro-muscular activity. *J. exp. Biol.* **31**, 260–270.

HOYLE G. (1955) The effects of some common solutions on neuromuscular transmission in insects. *J. Physiol.* **127**, 90–103.

HUDSON A. (1958) The effect of flight on the taste threshold and carbohydrate utilization of *Phormia regina* Meigen. *J. Ins. Physiol.* **1**, 293–304.

ROBERTSON J. D. (1957) *Recent Advances in Invertebrate Physiology* (Edited by B. T. SCHEER), pp. 229–246. University of Oregon Press.

WELLINGTON W. G. (1948) The light reactions of the spruce budworm, *Choristoneura fumiferana* Clemns (Lepidoptera, Tortricidae). *Canad. Ent.* **80**, 56–82.

WIGGLESWORTH V. B. (1934). The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera) —II. Factors controlling moulting and metamorphosis. *Quart. J. micr. Sci.* **77**, 191–222.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 38 *to* 44. *Pergamon Press Ltd., London. Printed in Great Britain*

ON THE OSMOTIC REGULATION OF THE LARVAE OF *EPHYDRA CINEREA*

HARALD NEMENZ

II. Zoologisches Institut der Universität, Wien, Austria
and
Department of Experimental Biology, University of Utah, Salt Lake City, Utah, U.S.A.

(*Received* 1 *April* 1959)

Abstract—Investigation of the pressure of the larvae of *Ephydra cinerea* showed that the animals are able to maintain an internal pressure of 20·4 atm, the highest yet found in insects, over a wide concentration range at low temperatures (3°C). The variability is greater at higher temperatures (21°C), where the larvae adjust somewhat to the low pressure of the external medium (internal pressure drops down to 13·5 atm). Ligated larvae show somewhat poorer ability to regulate, an effect that is increased at higher temperature.

INTRODUCTION

Due to the high salt concentration of the Great Salt Lake in Utah (U.S.A.) only very few species of organisms can live in its waters. With the exception of certain algae, bacteria, and protozoa, one finds only the larvae of one fly, *Ephydra cinerea* Jones, 1906, and the brine shrimp *Artemia salina* (L.). All of these organisms must have some special adaptations to regulate their osmotic pressure and ionic balance to withstand the high salt concentration of the lake, which may rise to more than 32 per cent by weight (250 atm). The regulatory mechanisms of *Artemia* have been investigated by a number of workers (e.g. Plattner, 1957; Croghan, 1958). The only investigations on *Ephydra* larvae, however, were on species living in brackish or slightly salty water. For example, Ping (1921) worked on *Ephydra subopaca* (= *riparia*) whereas Beyer (1940) studied three European species, *E. riparia*, *E. micans*, and *Caenia fumosa*. There do not appear to be any previous reports on the osmotic regulation of larvae of *Ephydra* species occupying such an extremely saline environment as the Great Salt Lake.

MATERIAL AND METHODS

The larvae were collected at the southeast end of the Great Salt Lake near Saltair and were kept in lake water (density 1·20) in the refrigerator at 3°C until used. During this time they had access to food (algae, collected at the same place). Only third instar larvae were used in experiments. They were not fed during experiments since the normal food was difficult to accommodate to the varying salt concentrations and since the salt content of the food might influence the results. In the experiments the larvae were placed in lake water of different concentrations

for a period of 48 hr, since preliminary experiments showed that exposures of this duration were necessary to attain a steady state in the experiments with diluted lake water at 3°C (exposures longer than 48 hr showed the same results). The other experiments were carried out in the same time to attain uniformity. BEYER (1940) also found with his species that similar periods were required to complete equilibrium. After exposure to the test solution the larvae were blotted with absorbent tissue, rinsed in distilled water, and carefully blotted again. The haemolymph was then withdrawn directly from the heart with a very fine-tipped glass capillary. After the blood was withdrawn, the tip was plugged with plasticine, the other (empty) end was sealed with a microburner. The capillaries were then centrifuged to bring the liquid to the sealed end. Particles were also sedimented in this procedure. These particles did not noticeably influence the results. The capillaries were then quick-frozen in dry ice and stored on dry ice until processed.

The osmotic pressure of the haemolymph was determined by measuring the freezing-point depression using the method of GROSS (1954). These data were converted to atmospheres with the tables of WALTER (1936). The references were sodium chloride solutions of known concentration which were handled in exactly the same way as the haemolymph samples. Repeated freezing and thawing of the haemolymph was found to increase the variability of the results. The heat-sealing of the capillary had to be done very carefully since the thickness of the glass at the sealed end apparently influenced the rate of thawing of the sample.

VOL. 4 1960

EXPERIMENTS

1. *The osmotic pressure of living larvae in different media*

In experiments at low temperature (3°C) one finds that the living uninjured larvae can maintain their inner osmotic pressure over a wide range of variation in the external medium. Normal animals from the lake have an internal pressure of about 20 atm (24 determinations, mean $\pm \sigma/\sqrt{N}$: $20{\cdot}5 \pm 0{\cdot}77$). The individual values vary slightly in contrast to the findings of BEYER (1940), who could not find any variations in his samples. A few individual larvae had a much higher pressure than others (maximum 37·9 atm). It is possible that these larvae were moribund and could no longer regulate as well as fully active larvae. It will be shown later that defective larvae lose their ability to regulate. The internal pressure is very high, but is still less than 10 per cent that of the lake water (approximately 250 atm) and is about the same as that of *Artemia salina* from comparable environments (PLATTNER, 1957).

In dilutions of lake water : tap water of 1 : 1 and 1 : 3, there is essentially no change in the internal osmotic pressure since the means are statistically not different (Table 1). The regulatory mechanism operates in a hypotonic environment as well as in one which is hypertonic. The internal osmotic pressure even in tap water and redistilled water at 3°C is the same as in lake water.

The same experiments repeated at 21°C show different results. The internal pressure in lake water is the same, but with dilutions of the external medium a

decrease of the osmotic pressure of the haemolymph occurs (Table 2). It is interesting that the three intermediate osmotic pressures (1 : 1 and 1 : 3 lake water : tap water, and tap water) had essentially the same effect on the internal osmotic situation. There is no statistical difference between these values and that

TABLE 1—MEANS OF THE OSMOTIC PRESSURE OF THE HAEMOLYMPH OF UNINJURED LARVAE IN VARIOUS MEDIA AT 3°C

Medium	No. det.	Mean (atm)	Δ°C	Density of medium	Osmotic pressure of medium
Lake water	24	20·5 ± 0·77	−1·89	1·20	250
Lake water : tap water 1 : 1	8	20·4 ± 0·19	−1·88	1·11	115
Lake water : tap water 1 : 3	8	20·0 ± 0·37	−1·67	1·05	62
Tap water	8	19·6 ± 0·27	−1·63	1·005	—
Redistilled water	8	20·1 ± 0·21	−1·67	—	—

TABLE 2—MEANS OF THE OSMOTIC PRESSURE OF THE HAEMOLYMPH OF UNINJURED LARVAE IN VARIOUS MEDIA AT 21°C

Medium	No. det.	Mean (atm)	Δ°C
Lake water	8	21·4 ± 0·81	−1·78
Lake water : tap water 1 : 1	8	16·8 ± 0·73	−1·40
Lake water : tap water 1 : 3	8	17·8 ± 0·88	−1·48
Tap water	8	15·8 ± 1·39	−1·31
Redistilled water	8	13·5 ± 0·41	−1·12

for the lake water, but they are lower and more on one level. In redistilled water the internal osmotic pressure decreases further. Although the curve decreases, the *t*-test shows no significant differences between the values in redistilled water and tap water, probably due to the high variation of the values in tap water.

2. *The osmotic pressure of ligated larvae in different media*

The above experiments show that under certain conditions there is a movement of water and/or salts into or out of the larvae in response to osmotic stress. The site of such movement in this species has not been previously established. It is known that the anal papillae as well as the rectum of mosquito larvae can function as osmoregulators (e.g. RAMSAY, 1950; WIGGLESWORTH, 1938). On the other hand, insect cuticle shows some permeability to water and salts (ALEXANDROV, 1934; EIDMANN, 1922; GHILAROV and SEMENOVA, 1957; RICHARDS, 1951, pp. 298–319; 1957).

The relative role of the anal papillae and rectum on one hand and the general body surface on the other hand in the osmoregulation of *Ephydra cinerea* was studied with so-called ligated larvae.

For these experiments the larvae were ligated with a nylon thread behind the cerebral ganglia and in front of the last pair of prolegs before they were put into the different solutions. By this technique the cuticle was made the only site of exchange. The experiments at 3°C show that the cuticle alone is not able to maintain the internal osmotic pressure at varying external solutions since the ligated larvae adjust partly to the external solutions (Table 3). In hypertonic

TABLE 3—MEANS OF THE OSMOTIC PRESSURE OF THE HAEMOLYMPH OF LIGATED LARVAE IN VARIOUS MEDIA AT 3°C

Medium	No. det.	Mean (atm)	Δ°C
Lake water	8	41·4 ± 1·42	−3·45
Lake water: tap water 1:1	8	32·1 ± 1·73	−2·67
Lake water: tap water 1:3	8	31·0 ± 1·17	−2·58
Tap water	8	19·9 ± 0·62	−1·57
Redistilled water	8	13·7 ± 0·78	−1·14

solutions the larvae lose most of their turgidity, so that it is sometimes difficult to get sufficient haemolymph for measurements. In hypotonic solutions, the larvae become so turgid that they cannot move any more. It would appear, therefore, that the cuticle is slightly permeable to water but almost impermeable to salts.

TABLE 4—MEANS OF THE OSMOTIC PRESSURE OF THE HAEMOLYMPH OF LIGATED LARVAE IN VARIOUS MEDIA AT 21°C

Medium	No. det.	Mean (atm)	Δ°C
Lake water	8	60 ± 3·5*	−5·5
Lake water: tap water 1:1	8	58 ± 3·9*	−4·8
Lake water: tap water 1:3	8	39·5 ± 10·6*	−3·29
Tap water	8	16·2 ± 0·21	−1·35
Redistilled water	8	16·7 ± 1·13	−1·39

* These high values are only approximate because of the extreme extrapolation required.

But the permeability even for water is not very great since there has to be a fairly high concentration difference to get a noticeable flux of water through the cuticle at these low temperatures. This may explain why the osmotic pressure of the

larvae in tap water is not much different from the normal osmotic pressure. The values for larvae in redistilled water are much lower and show a statistically significant difference from the values in all of the other media including tap water (t-test, $P = 0{\cdot}035$).

The effects of external osmotic pressure were even greater in similar experiments carried out at a higher temperature (21°C), as shown in Table 4. Not expected, however, was the seeming increase in redistilled water. However the values for ligated larvae at 3°C and 21°C are not significantly different ($P = 0{\cdot}25$) from one another.

The internal osmotic pressure of ligated larvae immersed in lake water increases much more in experiments at 21°C than at 3°C as shown in Tables 3 and 4. This probably results from a change in the permeability of the cuticle. As MEAD-BRIGGS (1956) showed, the permeability of insect cuticle increases with rising temperature even at temperatures too low to melt the cuticular lipids. The change in the permeability with temperature is more or less continual and is probably due to the fact that the various single lipids have different melting points so that the mixture does not have a sharp melting point. The argentaffin reaction shows that the cuticle of larval *Ephydra cinerea* possesses a well-defined lipid layer. This would explain why the permeability increases with rising temperatures.

DISCUSSION

The results show that the adaptation of *Ephydra cinerea* to highly concentrated salt solutions is only partly due to a rather impermeable cuticle and probably partly to an active regulation. The fully active animals regulate their internal concentration to an optimal value or at least to a bearable one. In lake water, where the concentration gradient is extremely high, the internal concentration is higher than in dilute media. The optimal inner concentration seems to be at about 17 atm, because this is maintained across a wide range of external concentrations. It is likely that the impermeability of the cuticle is a more important factor in osmoregulation at lower temperatures, as the behaviour and mode of life of the larvae is different at these temperatures (NEMENZ, 1959). On the other hand the active regulation has to be effective enough to ensure the vital low concentration in summer.

The larvae must be able to withstand a range of external concentrations since the lake water is more dilute in spring than it is in winter. It is doubtful that natural dilutions up to 1 : 1 would ever occur, however (FLOWERS, 1934; NEMENZ, 1959). The local variations may be higher than those of the lake proper, but will not influence the bottom inhabiting larvae so much. The high values found for the normal internal osmotic pressure of the larvae are very surprising and do not agree at all with those reported by BEYER (1940). The published data on *Ephydra riparia* correspond to an internal osmotic pressure of 4·9–6·9 atm, about $\frac{1}{4}$–$\frac{1}{3}$ of *E. cinerea*. In fact the values presented here for *E. cinerea* are the highest internal osmotic pressures ever reported for an insect ('water beetles' 12·16 atm, WIGGLESWORTH, 1947, p. 230; *Tenebrio molitor* larvae 14·1 atm, ROUSCHAL,

1940; *Tenebrio molitor* larvae 16 atm, ROEDER, 1953, p. 149), but they are still slightly lower than the osmotic pressure of sea water (about 22 atm) and about as high as the osmotic pressure of *Artemia salina*. This high internal osmotic pressure reduces the difference between external and internal medium. It is very likely that the differences in the osmotic values and their regulation, compared with the other investigated *Ephydra* species represent, an adaptation and are largely due to the very high concentration of the Great Salt Lake.

The changes observed in ligated larvae indicate that the cuticle is more permeable to water than to salts, and that the influx and outflux of a given substance can be different. The absence or presence of metal ions in the environment, especially Ca^{2+}, possibly influences the permeability of the cuticle to the point where active regulation can no longer prevent changes. Experiments to clarify this are under way.

SUMMARY

(1) The osmotic pressure of the haemolymph of the larvae of *Ephydra cinerea* living in the Great Salt Lake is about 20·4 atm, the highest yet found in insects.

(2) Living uninjured larvae are able to maintain the internal pressure at low temperatures (3°C) over the concentration range from lake water to redistilled water.

(3) At higher temperatures (21°C) the larvae adjust to a somewhat lower pressure (about 17 atm) in 50 per cent lake water–fresh water and to an even lower value (about 13·5 atm) in distilled water.

(4) Larvae ligated at both ends so that materials could penetrate only through the cuticle show somewhat poorer ability to regulate. This effect is increased at higher temperatures.

(5) The osmotic regulation is probably accomplished by a very impermeable cuticle in addition to an active regulation mechanism.

Acknowledgements—This work was carried out in the Department of Experimental Biology, University of Utah, Salt Lake City, and thanks are due to Dr. JOHN D. SPIKES for providing the necessary facilities. The study was made possible by an appointment supported by the International Cooperation Administration under a programme administered by the National Academy of Sciences. For the leave of absence from the University of Vienna, Austria, the author would like to thank Prof. Dr. W. KÜHNELT, as well as for his help in arranging the appointment.

REFERENCES

ALEXANDROV W. (1934) Die Permeabilität des Chitins einiger Diptera-larven und eine Methode für Permeabilitätsforschungen. (Russian, German summary.) *Biol. Zh.* **3**, 490–507.

BEYER A. (1939) Morphologische, ökologische und physiologische Studien an den Larven der Fliegen *Ephydra riparia*, *E. micans* und *Caenia fumosa*. *Kieler Meeresforsch.* **3**, 265–320.

CROGHAN P. C. (1958) The osmotic and ionic regulation of *Artemia salina* L. *J. exp. Biol.* **35**, 219–233.

VOL. 4 1960

EIDMANN H. (1922) Die Durchlässigkeit des Chitins bei osmotischen Vorgängen. *Biol. Zbl.* **42**, 429–435.

FLOWERS S. (1934) Vegetation of the Great Salt Lake region. *Bot. Gaz.* **95**, 353–418.

GHILAROV M. S. and SEMENOVA L. M. (1957) Die Kutikelpermeabilität bodenbewohnender Tipulidenlarven. *Z. PflKrankh.* **64**, 522–528.

GROSS W. J. (1954) Osmotic responses in the sipunculid *Dendrostomum zostericolum*. *J. exp. Biol.* **31**, 402–423.

MEAD-BRIGGS A. R. (1956) The effect of temperature upon the permeability to water of arthropod cuticles. *J. exp. Biol.* **33**, 737–749.

NEMENZ H. (1959) Zur Biologie von *Ephydra cinerea*. *Zool. Anz.* In press.

PING C. T. (1921) The biology of *Ephydra subopaca* Loew. *Mem. Cornell agric. Exp. Sta.* **49**, 555–616.

PLATTNER F. (1955) Der osmotische Druck von *Artemia salina*. *Pflüg. Arch. ges. Physiol.* **261**, 172–182.

RAMSAY, J. A. (1950) Osmotic regulation in mosquito larvae. *J. exp. Biol.* **27**, 145–157.

RICHARDS A. G. (1951) *The Integument of Arthropods*. University of Minnesota Press, Minneapolis.

RICHARDS A. G. (1957) Studies on arthropod cuticle—XIII. The penetration of dissolved oxygen and electrolytes in relation to the multiple barriers of the epicuticle. *J. Ins. Physiol.* **1**, 23–39.

ROEDER K. D. (1953) *Insect Physiology*. Wiley, New York.

ROUSCHAL W. (1940) Osmotische Werte wirbelloser Landtiere und ihre ökologische Bedeutung. *Z. wiss. Zool.* **153**, 196.

WALTER H. (1936) Tabellen zur Berechnung des osmotischen Wertes von Pflanzensäften, Zuckerlösungen und einigen Salzlösungen. *Ber. dtsch. bot. Ges.* **54**, 328–339.

WIGGLESWORTH V. B. (1938) The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae *J. exp. Biol.* **15**, 235–247.

WIGGLESWORTH V. B. (1947) *The Principles of Insect Physiology*. Methuen, London.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 45 *to* 62. *Pergamon Press Ltd., London. Printed in Great Britain*

ENDOGENOUS NERVE ACTIVITY AND BEHAVIOUR IN THE MANTIS AND COCKROACH*

KENNETH D. ROEDER, LILLIAN TOZIAN,† and ELIZABETH A. WEIANT

Department of Biology, Tufts University, Medford 55, Mass., U.S.A.

(*Received* 6 *April* 1959)

Abstract—A decapitated male mantis makes co-ordinated movements of the legs, abdomen, and phallomeres. These movements continue for days and lead to rapid and effective copulation if a female mantis is encountered. The efferent nerve activity responsible for some of these movements is shown to be endogenous, continuing after all afferent connexions of the ganglion have been cut. This motor nerve activity is normally controlled by inhibition via descending pathways from centres in the sub-oesophageal ganglion. Many efferent neurons in the last abdominal ganglion and the metathoracic ganglion of the cockroach show similar endogenous rhythmic activity, and are likewise controlled by inhibition from other centres. The pattern of endogenous activity and the nature of the inhibition have been explored, although the behavioural significance of this system is less clear in the cockroach than in the mantis. The possible phylogeny and general significance of an endogenously active motor system controlled by parasegmental inhibition is examined and compared with the more generally recognized stimulus-response motor mechanism.

VOL. 4 1960

INTRODUCTION

Most efforts to analyse the behaviour of animals and of the neurons that make up their nervous systems have been based on the method of applying controllable stimuli and measuring the resulting responses. The intact living animal offers us an inscrutable 'black box' and the most direct approach is to tamper with the inputs and record changes in the outputs.

This method reveals only stimulus-response relations and the picture that emerges from its application is naturally one of a stimulus-response system. In other words, we are forced *a priori* to the assumption that the animal is a stimulus-response system in order to justify carrying out the experiment. However, it is becoming increasingly evident that this view is insufficient to encompass the behaviour of animals, particularly when they are studied under conditions less restricting and more natural than those provided by the usual laboratory stimulus-response situation (Lorenz, 1950; Tinbergen, 1951; reviews by Roeder, 1955; Eibl-Eibesfeld and Kramer, 1958).

* This work was supported by grants from the U.S. Public Health Service and the National Science Foundation. Some of the equipment was purchased under a previous contract between The Chemical Corps U.S. Army and Tufts University.

† Work on the last abdominal ganglion of the cockroach was submitted in partial fulfilment of the requirements for the M.A. degree at Tufts University, 1957.

In order to explain the observations made by these and other workers it is necessary to assume that behaviour is not merely a reaction to a specific and current stimulus situation, but may also be spontaneous or endogenous. This means that the onset of some behaviour patterns is an outward expression of the intrinsic organization and instability of neurons within the central nervous system, and that such patterns are theoretically capable of appearing under conditions of zero sensory input. If this is so, sensory input might be expected either to augment or to suppress the behaviour in question. The postulated situation is analogous to the well-known endogenous myogenic rhythm of the vertebrate heart beat. With all nerve connexions cut, the heart continues to beat; vagal nerve impulses depress the heart's spontaneous tendency while its beat is augmented by sympathetic impulses. At the same time, its 'normal' spontaneous tendency is influenced by the chemical composition of the fluids in which it is bathed.

It must be emphasized that in this paper endogenous or spontaneous activity is defined as that which can be shown to occur in the absence of sensory input to the central nervous system. The adjectives 'endogenous' and 'spontaneous' are used interchangeably although 'endogenous' seems to be more suitable in the present context. However, most references in the literature to activity in de-afferented nervous systems characterize it as 'spontaneous'. Internal chemical states and regenerative feedback mechanisms as well as intrinsic organization and diffuse or specific previous experience must all be important in determining the presence or level of the unstable state of central neurons, but it seems legitimate to consider these factors as a separate class from the immediate afference due to stimuli coming from outside the organism. These aspects of the question have been discussed elsewhere (ROEDER, 1955) and the object of this paper is to present and discuss evidence of spontaneity in certain aspects of insect behaviour and central nervous activity.

Experimental demonstration of endogenous activity encounters a serious practical difficulty imposed by the condition of zero sensory input. Direct demonstration of spontaneity seems to demand this condition, yet it is difficult, if not impossible, to devise an experimental situation of zero sensory input containing a normal experimental animal. This places the concept of spontaneity in behaviour at a considerable heuristic disadvantage, and the experimental evidence that can be assembled is much more indirect and circumstantial than that available from studies of animals in stimulus-response situations.

LOCOMOTOR BEHAVIOUR IN THE PRAYING MANTIS

The need to supplement the stimulus response concept with the approach outlined above is illustrated by an examination of the factors controlling locomotion in the praying mantis.

Initial observations were made on *Mantis religiosa* L. (ROEDER, 1937) and were confirmed and extended more recently on *Heirodula tenuidentata* Saussure. The latter were originally supplied by Dr. HORST MITTELSTAEDT (Max-Planck-Institut

für Verhaltensphysiologie, Germany) and have been in continuous culture in this laboratory for about three years.

Mantids are capable of a wide variety of locomotor patterns, but there are certain conditions under which they remain motionless for long periods of time. A single individual placed in a large cage at 27°C, illuminated by fluorescent light for 12 hr per day, and provided with a steady food supply of living flies, may remain in the same position upside-down on the cage roof for several days. There seems to be no diurnal locomotory rhythm and the only observed actions are occasional grooming movements and strikes at flies moving within reach. The situation seems to be optimal and the insect stays there. The nature of this optimal situation has not been defined, but it requires an inverted posture and generally a region of fairly high light intensity. Since the form and colour of these mantids is procryptic and they feed by capturing insects alighting nearby (RILLING *et al.*, 1959), the adaptive value of immobility is fairly obvious. Locomotor activity in mantids deprived of food, in unfertilized females, and in mantids approached by a potential predator (CRANE, 1952) will not be discussed here.

VOL. 4 1960

At first sight this might be taken for a simple stimulus-response situation—no stimulus change therefore no response. That the situation is more complex than this is shown by surgical removal of the brain (supra-oesophageal ganglion). After this is done mantids at any stage and under any condition walk steadily and continuously in a perfectly co-ordinated manner (ROEDER, 1937). Walking usually continues to exhaustion or until the insect has become so entangled in branches that further forward movement is impossible.

Removal of the brain entails loss of the special sense organs (antennae, ocelli, and compound eyes) in addition to nerve tissue. If these sense organs are removed separately or collectively, leaving the brain intact, there is no departure from the normal tendency to immobility, and it was concluded that the supra-oesophageal ganglion continuously suppresses excitatory locomotor centres in the sub-oesophageal ganglion (ROEDER, 1937), and that the special sense organs have little influence on general activity or immobility. Therefore, the normal immobility of a mantis in ambush must depend upon continuous activity of the locomotor inhibitory centres in the brain. When locomotion occurs, the spontaneous tendency of lower centres to cause rhythmic leg movements must be released from this inhibition.

The flaw in the surgical experiment with the brainless mantis is that although certain special sense organs have been eliminated as factors in locomotion, a multitude of other receptors on the body surface are providing input to the remaining ganglia of the ventral nerve cord. The continuous locomotion that follows debraining could be ascribed to reflex actions from this multichannel input, and it cannot be proved whether activity is due to this reflex mechanism or to an endogenous output from the nervous system. No surgical procedure has been designed that can eliminate this input without also preventing motor impulses from reaching the muscles and hence making all behaviour impossible.

THE SEXUAL BEHAVIOUR OF THE MALE MANTIS

A somewhat more complete example of the role of spontaneity in behaviour is provided by an analysis of the courtship activities of the male mantis.

Cannibalism among the later larvae and adults is fairly common in captivity. Its incidence in nature is unknown, but it is undoubtedly lower when the insects are more thinly distributed. Cannibalism seems neither to be completely indiscriminate nor does it occur only under conditions approaching starvation. The factors concerned are under study and will be reported in a later paper.

A special opportunity for cannibalistic attack that must also exist in nature is provided by the proximity of male and female at the time of mating. This has been described in detail for *Mantis religiosa* (ROEDER, 1935) and will be summarized. The situation in *Heirodula tenuidentata* is essentially similar and has provided additional information.

The male is smaller and more lightly built and has larger eyes and longer antennae. When mature he reacts visually to the movements of a nearby female by making following movements of the head and by freezing. Movements of the female release in the male very slow and frequently interrupted approach movements. This slow and 'stealthy' approach may be made from any angle, during which the male shows no primary signs of sexual excitation. When the male is about one length away he mounts the female in a quick movement involving a combination of flying, leaping, and running.

During his approach the immobility and slow movements of the male rarely elicit any response from the female, but there are two situations that expose the male to cannibalistic attack. First, if the male has not detected the presence of the female, but moves near her he may be grasped and eaten. Second, when the male lands on the female's back he may do so at any angle. The female may react violently to the impact of the male's arrival, reaching back with her forelegs and beginning to eat that part of the male (usually his head) that is nearest. On the other hand, once the male is in position on the back of the female he is rarely molested.

Attack on the male is not inevitable, and some males have been observed to mate 5 or 6 times with the same or with different females. Often the female remains completely passive as the male mounts her, and lack of food, sexual readiness, and possibly other factors determine the outcome. After the male is in position the female may either remain motionless or move about. The male remains motionless on her back, or turns quickly around if he has landed in the reversed position. For 10–30 min he shows little or no sexual activity although he occasionally flagellates the female with his antennae. At the end of this preliminary period the male begins to make search movements with the abdomen, bending it in a characteristic S shape (Fig. 1 a). This leads eventually to connexion of the phallomeres with the ovipositor and the deposition of a spermatophore, a process that may take some hours.

When the male is attacked in either of the two situations described above, consumption of his head by the female is followed by rotary locomotor movements

VOL.
4
1960

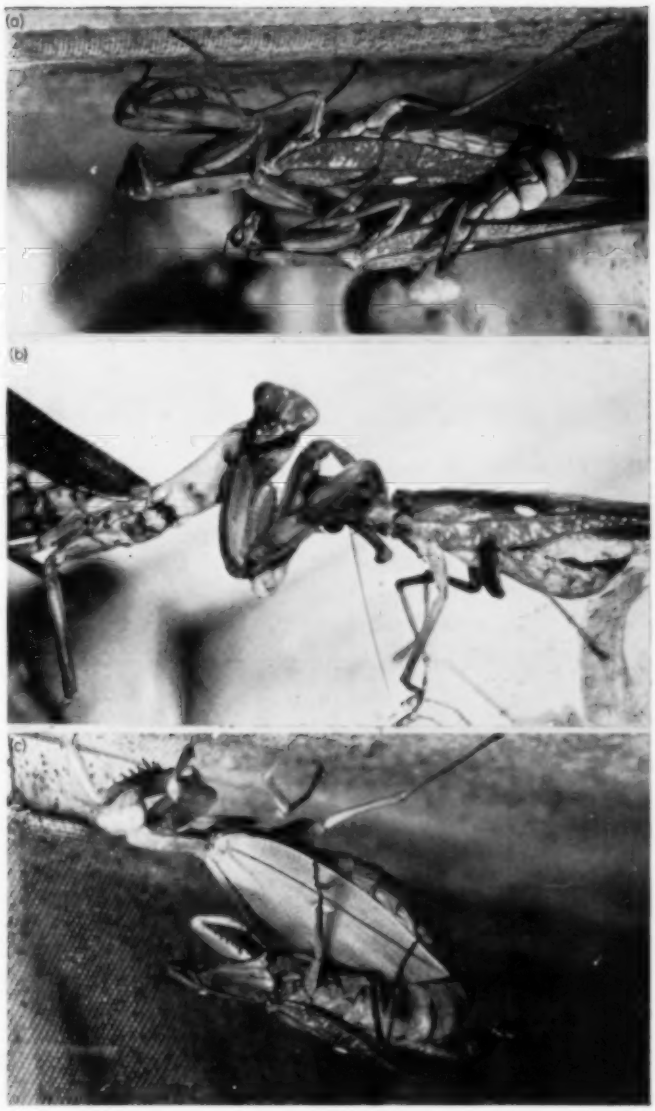


FIG. 1. Sexual behaviour in *Heirodula tenuidentata*. The insects normally hang upside-down. (a) Copulating pair in which the female did not attack the male. (b) A female has attacked an approaching male. She has bitten through the nerve-cord in the pro-thorax and the male abdomen already shows the bending characteristic of copulation. (c) Decapitated male in successful copulation.

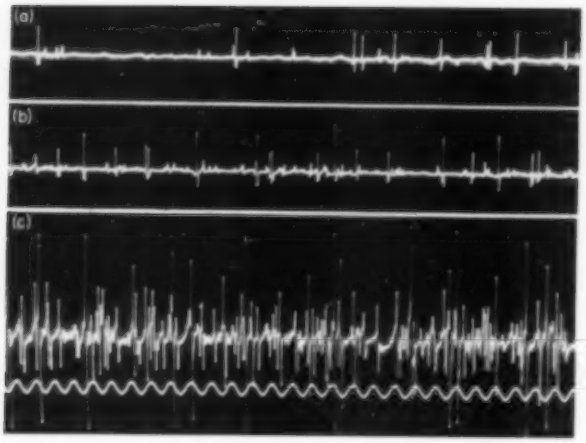


FIG. 3. Efferent impulses in nerve X of male *Mantis religiosa*. The nerve has been cut distal to the electrodes, and all other nerves arising from the ganglion have been severed. (a) Before transection of the abdominal connectives. (b) 3 min after cord transection. (c) 7 min after cord transection. Time line 100 c/sec.

and vigorous search movements by the male abdomen (Fig. 1 b). The rotary movements frequently carry the male onto the female's back if he is attacked before mounting her, while the S-movements of the abdomen lead to more rapid copulation (Fig. 1 c) than occurs when the male is intact.

Analysis of this situation by the surgical removal of various ganglia showed that a nerve centre in the sub-oesophageal ganglion (in the head) exerts a continuous inhibitory action upon centres in the thoracic ganglia and last abdominal ganglion (ROEDER, 1935). The latter contains the motor centres for the rotary locomotor movements and the copulatory movements of the abdomen respectively. This neural arrangement has obvious adaptive value since the male is liable to attack by the female prior to copulation. It is interesting to note that the copulatory movements of a decapitated male are more intense than those of an intact male, and may continue without pause for several days irrespective of whether or not the male is in contact with a female. In securing fertilized eggs for the mantis culture it has sometimes been necessary to decapitate the males and place them on the backs of the females when the intact males showed little tendency to court.

VOL. 4 1960

The rotary locomotor movements have never been observed in intact males, while the S-shaped bending of the abdomen appears only during the later stages of courtship. Since both these actions must be continuously suppressed by the sub-oesophageal centre during most or all of the adult male's life it seems probable that they are produced by an organized pattern of efferent nerve impulses dependent only upon the organization and functional states of neurons within the central nervous system. The sensory pattern derived from contact of a mature male with a female would then be expected to reduce or negate this inhibitory action of the sub-oesophageal ganglion. An alternative hypothesis based on the stimulus-response concept might be that the sub-oesophageal centre normally prevents sensory impulses from reaching appropriate reflex centres in the ventral ganglion chain. Decapitation would then remove this block. The only direct way of testing these hypotheses would be to examine the behaviour of the male after elimination of all sensory input either by placing the animal in a 'zero' environment or by cutting all afferent pathways. Both methods are impracticable, but a close approximation to this objective was attained by the following electrophysiological experiment.

INHIBITION OF MOTOR IMPULSES IN THE LAST ABDOMINAL GANGLION OF THE MALE MANTIS

Movements of the phallomeres and the S-bending of the abdomen observed during copulation persist even in an isolated abdomen, and their motor origin can be traced to the last abdominal ganglion. This ganglion is in reality the fused ganglia of abdominal segments VII to XI and distributes nerves to the general musculature of these segments, the phallomeres, and the cerci (Fig. 2).

Insect nerves are not divided into dorsal sensory and ventral motor roots as in vertebrates, and there is no surgical way in which afference can be eliminated without also eliminating motor fibres to the muscles, and hence behaviour. However, it

is possible to place recording electrodes below the nerves leaving the ganglion. A suitable amplifier and oscilloscope can then be used to display the afferent and efferent nerve impulses passing the electrode site. Transection of the nerve distal

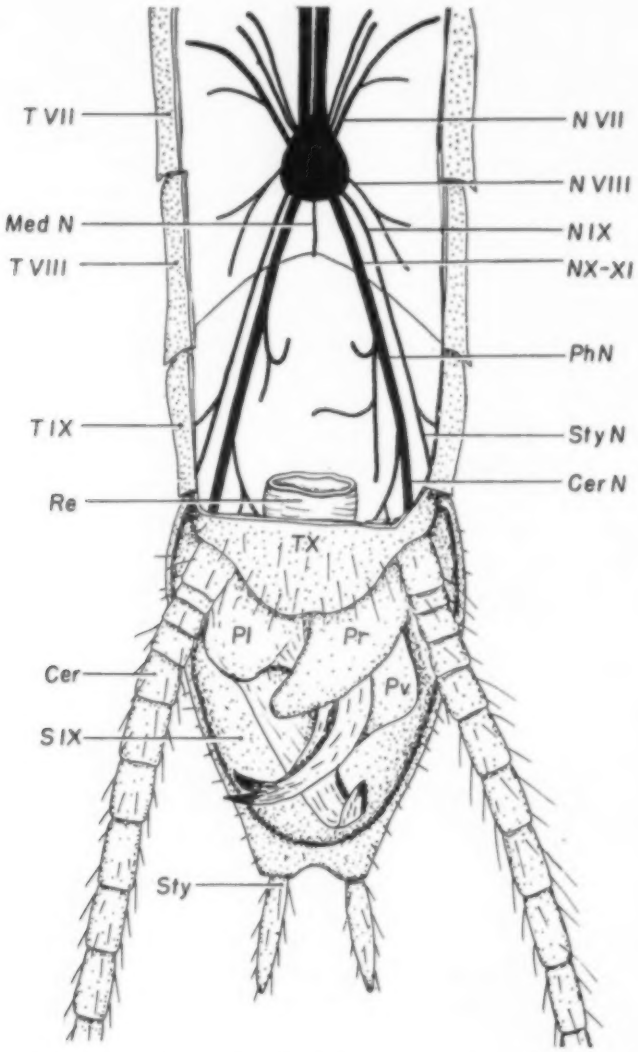


FIG. 2. Dorsal view of the last abdominal ganglion and terminal segments in male *Mantis religiosa*. The nerves are numbered according to the abdominal segments they supply. Cer, cercus; CerN, cercal nerve; MedN, median nerve; N VII to N XI, segmental nerves of last abdominal ganglion; PhN, phallic nerve; Pl, Pr, Pv, left, right, and ventral phallomeres; Re, rectum; S IX ninth sternum; Sty, stylus; StyN, stylus nerve; T VII to T IX, tergites.

to the electrodes eliminates all afferent nerve impulses at the electrode site and also prevents any afferent impulses from reaching the ganglion in fibres of that particular nerve. The remaining impulses must all be efferent and would have been manifest behaviourally as some degree of muscle tension or contraction if the nerve had not been transected.

An adult male mantis was pinned dorsal side up on a cork plate and the abdominal nerve cord was exposed by removal of the tergites, digestive tract, and gonads. Electrodes were placed below the phallic nerve (Fig. 2) which was then transected distally. All other nerves arising from the last abdominal ganglion were also transected, thereby eliminating the possibility of afferent impulses reaching the ganglion with the exception of those that might arrive via internuncial fibres in the paired connectives joining the ganglion to the rest of the ventral nerve cord and brain.

The efferent nerve activity recorded in the phallic nerve under these circumstances commonly took the form of a fairly regular sequence of small spikes in several fibres (Fig. 3 a). Occasionally this steady discharge was modulated by bursts of impulses related to respiratory movements of the abdomen (innervated by more anterior ganglia in the chain) and by erratic bursts coincident with movements of the insect. However, the same basic pattern could be followed for an hour or more if the preparation was kept moist with saline and in good condition.

VOL. 4 1960

When this activity remained at a fairly steady level the connectives just above the ganglion were transected. This operation eliminated the only connexion between the ganglion and the rest of the nervous system, including nerve centres in the head. Following a transitory burst of impulses due to injury, the activity in the phallic nerve continued for a short period at its previous level. Within 1–5 min there was a noticeable increase both in the number of active nerve fibres and in the frequency of spikes in previously active fibres (Fig. 3 b). This increase reached a maximum in 5–15 min (Fig. 3 c) and continued at the same level for an hour or more. The recording shows that this heightened activity included repetitive spikes in several large nerve fibres that had previously given no sign of activity. In some cases, the heightened efferent activity showed a marked rhythmic waxing and waning.

Transection of the connectives eliminated not only input from postulated inhibitory centres in the head but also the last possible source of sensory input to the ganglion. Therefore, the efferent discharge is spontaneous in the sense defined above. Since the phallic nerve contains motor fibres to the muscles of the phallomeres, it is hard to avoid the conclusion that this greatly increased and fluctuating output of efferent spikes in the isolated ganglion would have caused movements of the phallomeres had the phallic nerve not been transected distal to the electrodes. Hence, spontaneous movements of the phallomeres can occur.

Similar increases in motor activity were observed in other nerves supplying muscles of the abdominal wall that would normally be involved in the S-bending. Also, the same increase in motor activity occurred if the behavioural experiment was duplicated, i.e. the mantis was decapitated. The increase following

decapitation was not modified significantly if the connectives above the ganglion were subsequently transected. Therefore, the effects described above are largely or entirely due to elimination of connexions with the head of the insect, and sensory input from the rest of the body has no direct influence either on the inhibitory action or upon the subsequent increase.

This statement is not meant to suggest that sensory impulses play no part in the sexual behaviour of the mantis. It merely says that the behaviour can occur in the absence of sensory input. The role of sensory input becomes evident if we examine once more the behaviour of a decapitated male mantis. If the insect is placed on a plane surface it rotates almost continuously for days (ROEDER, 1935) while making probing S-movements of the abdomen and movements of the phallomeres. When an object such as a pencil or the body of another mantis is encountered during these gyrations, the rotary locomotion stops and the object is grasped strongly. However, the abdomen continues its movements unless the genitalia of a female mantis are encountered by the phallomeres. When this happens coupling takes place and the abdominal movements cease. Some specific sensory pattern arising from the appropriate contacts must modify or block the spontaneous action patterns, serving in this fashion to steer the behaviour.

Electrophysiological investigation of the origin of the rotary locomotion has not been made. However, it is clearly dependent upon a separate neural organization in the thoracic ganglia of the male. Amputation of the abdomen of a headless male modifies neither the rotary movements of the thoracic part nor the S-bending and phallomere movement of the abdominal part.

ENDOGENOUS NERVE ACTIVITY AND ITS INHIBITION IN THE COCKROACH

The observations and experiments reported above show that in the male mantis endogenous activity of neurons in the last abdominal ganglion is the source of an adaptively significant behaviour pattern. This led to a search for additional correlations between endogenous activity and behaviour and to a closer examination of the factors influencing the spontaneously active motor neurons and the inhibitory centres controlling them. Mantids are difficult to raise in quantity and were not always available. Therefore, much of the work was carried out on a related orthopteran, the American cockroach, *Periplaneta americana*.

Method

Adult male cockroaches were used in most of the experiments so as to provide a comparison with the situation in the mantis and also because dissection and exposure of nerves was easier than in females or larvae. The insects were restrained by pinning on a cork sheet, the appropriate ganglia and nerves were exposed by dissection, and fine hooked silver electrodes were placed under the nerves. Exposed portions of the nervous system were moistened or immersed in Hoyle's saline (HOYLE, 1953). Comparison of efferent nerve activity in the insect's own haemolymph and in Hoyle's saline showed no significant differences.

In most cases that portion of the nerve lifted above the saline surface was allowed to dry. Thus, it served merely as an electrical connexion between the electrode and the living portion of the nerve lying below the saline. This method (ROEDER and TREAT, 1957) has the advantage that activity in small nerves can be followed for long periods without the electrical disturbances caused by frequent irrigation. Slow changes in spike height occurred owing to changes in the series resistance of the drying nerve, but this stabilized at a satisfactory point provided the humidity of the air surrounding the preparation was not too low. The method requires a high-impedance cathode-follower input. Conventional resistance–capacity coupled amplifiers were used in conjunction with oscilloscopes, loud-speakers, tape recorders, and various integrating devices.

Endogenous activity in the last abdominal ganglion

The gross anatomy of the last abdominal ganglion of *Periplaneta* is similar to that of *Mantis religiosa* (Fig. 4; compare with Fig. 2). It is formed by the fusion of ganglia belonging to abdominal segments VII to XI. Nerves IX and X were most frequently used as sources of efferent activity. Nerve IX is mixed, sending motor branches to the general musculature of its segment and to certain muscles at the base of the cercus. Nerve X has two branches that were examined separately. The dorsal branch is mixed, containing afferent fibres from campaniform sensilla at the base of the cercus and efferent fibres to cercal muscles. The ventral branch of X, also called the phallic nerve, contains only motor fibres to various phallomere muscles.

At first the nervous system of the cockroach was left intact with the exception that the nerve used to provide efferent impulses was cut distal to the recording electrode. This eliminated afferent impulses over that particular nerve. Under these circumstances efferent fibres in the phallic nerve were commonly inactive (12 out of 14 cases). Nerve IX usually showed a regular discharge of small efferent spikes (47 out of 59 cases) that probably indicated a tonic state of the abdominal musculature. The dorsal branch of X was intermediate in its efferent activity.

Elimination of local sensory input by progressive transection of all other nerves arising from the ganglion caused no change in the output of the phallic nerve and IX. In the dorsal branch of X there was a small but consistent increase when the cercal nerve (XI) was cut. The cercal nerve contains afferent fibres from hair sensilla on the cercus and serves as the input for the evasion response mediated by the giant fibre system. The change in X (supplying some of the cercal muscles) suggests alteration in some local reflex system due to removal of its input. It was not investigated further, but was insignificant compared with the increases in efferent activity reported below.

Complete neural isolation of the last abdominal ganglion was accomplished by cutting the abdominal connectives joining it to the rest of the ventral nerve cord. This was followed by a marked and sustained increase in the efferent activity in IX and both branches of X (Fig. 5). The increase was maintained for more than 10 min in 23 out of 32 cases, for less than 10 min in 5 cases, and was doubtful in 4 cases. Commonly the increase in efferent impulses was apparent within 5 min

VOL. 4 1960

after transection of the cord, but its time of onset was quite variable, ranging from a few seconds to 15 min or more. Spontaneous activity usually reached peak level in an additional 10 min and continued in most preparations for several hours.

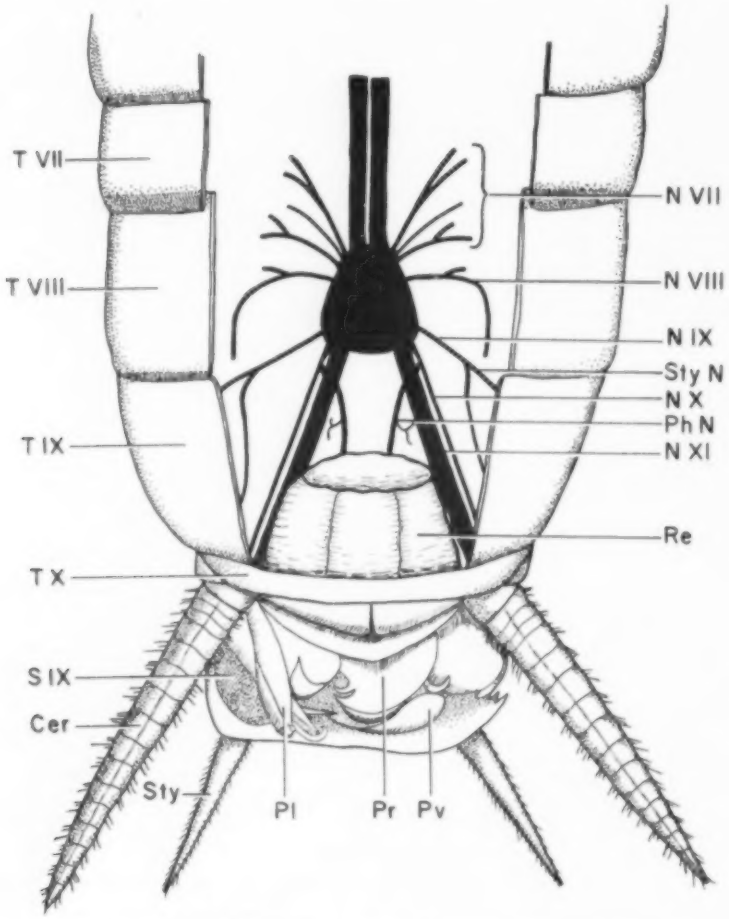


FIG. 4. Dorsal view of the last abdominal ganglion and terminal segments in male *Periplaneta americana*. Structures labelled as in Fig. 2.

The nature of the increase varied with the nerve chosen, but generally it was very similar to that recorded under like conditions in the mantis (Fig. 3). Small units that were already active showed an increase in frequency of firing (common in IX), and in all three nerves much larger spikes appeared in fibres that had previously been inactive. Sometimes the large spikes continued in a regular sequence for considerable periods. At other times, particularly in the phallic nerve,

VOL.
4
1960

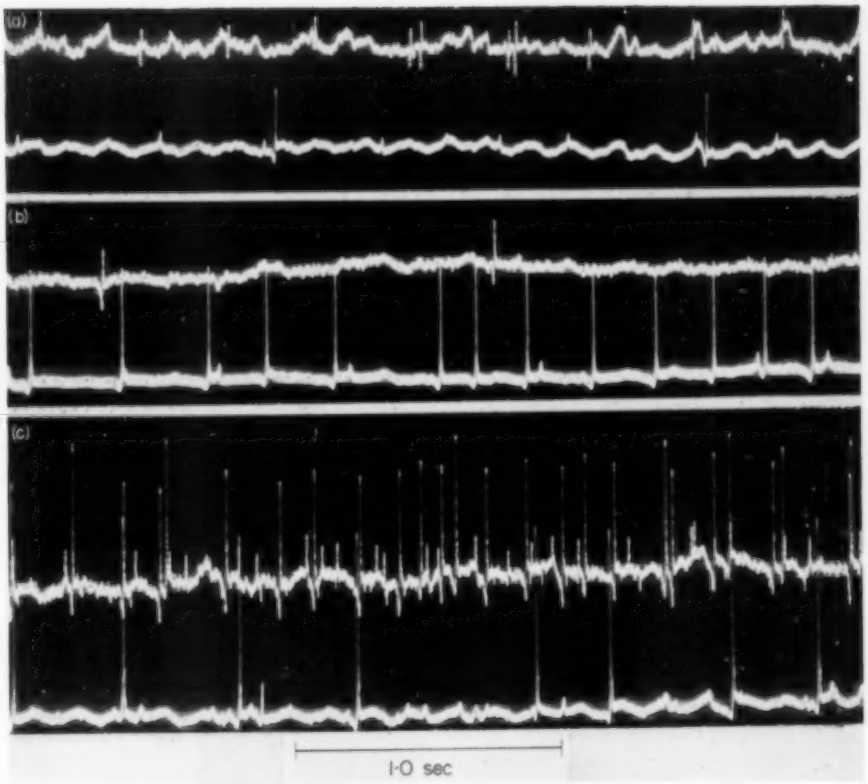


FIG. 5. Efferent nerve impulses in nerves IX (upper trace) and the phallic branch of X (lower trace) of male *Periplaneta americana*. (a) The abdominal connectives still join the ganglion to the rest of the nervous system. (b) 4 min after transection of the nerve cord. A large spike appears in the phallic nerve record. These spikes are usually grouped in bursts (see Fig. 6). (c) 10 min after transection of the nerve cord. In nerve IX one fibre that showed some activity in (a) is now firing more rapidly, while another fibre (larger spike) has only now become active. The large unit in the phallic nerve is firing more slowly than in (b), since the record coincided with an inter-burst period in this nerve. There is no synchrony between the active units in the two nerves.

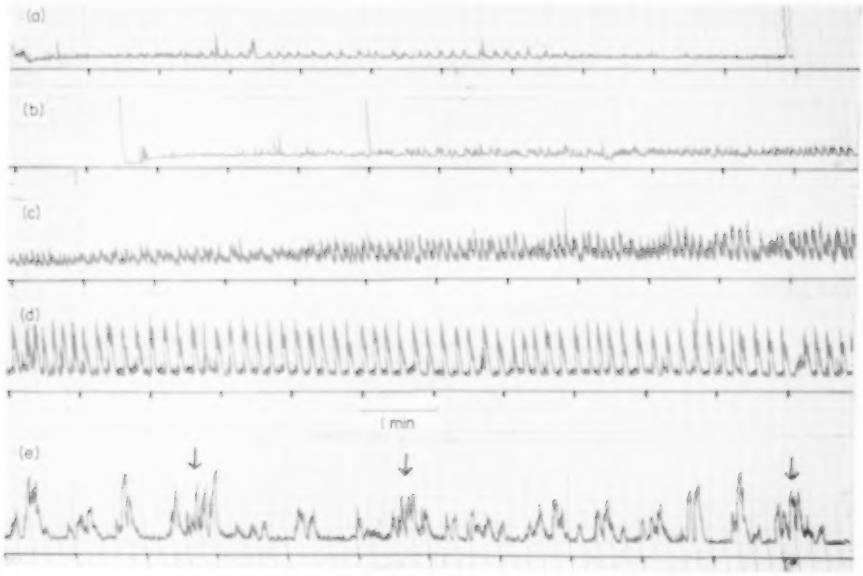


FIG. 6. Record of integrated spike activity in the phallic nerve of *Periplaneta americana*. Time marks below each record indicate 1 min intervals. (a) Activity when the last abdominal ganglion is still connected to the rest of the nervous system. (b) Continuous with (a). The insect was decapitated at the beginning of this section of the record. (c) Continuous with (b). (d) 36 min of recording has been omitted between (c) and (d). Rhythmic efferent bursts are now well developed. (e) Similar record from another preparation 50 min after decapitation. The pattern of efferent bursts is more complex, but rhythmic repetition of complex patterns (arrows) is still apparent.

the large spikes were grouped into regularly timed volleys. Part of one of these volleys is seen in Fig. 5 (c). Each volley lasted 1 or 2 sec and recurred several times a minute. This volleying pattern often continued for hours. The volleying pattern became more evident when the spike output of the phallic nerve was electronically integrated and the envelope of the integration recorded on an ink-writer at a writing speed much slower than that used in making the oscillograms (Fig. 6). Sometimes repeating volleys having several different rhythms were recorded simultaneously from different efferent fibres in the same nerve (Fig. 6 d). Transient volleying of the same kind was sometimes seen before the nerve cord was cut (Fig. 6 a), suggesting transient and partial release of efferent activity from the inhibition. Following transection of the nerve cord volleying then appeared strongly and permanently (Fig. 6 c).

Transection of only one of the paired abdominal connectives was usually followed by increased efferent activity in nerves on both sides of the ganglion. Bisection of the desheathed ganglion in the mid-line caused a sustained increase in the motor output that was even greater than that produced by transection of both abdominal connectives. This suggests mutual contralateral inhibition between the right and left halves of the ganglion (WEIANT, 1958) in addition to the possibility of decussation of the descending inhibitory pathways. However, the operation of bisection of the ganglion must be considered with some reservation. Not only must the ganglion suffer considerable mechanical damage in its central region, but this central region is exposed for the first time to the direct action of saline. Desheathing the ganglion brought about no change in the efferent activity.

VOL. 4 1960

Although efferent fibres arising from the last abdominal ganglion become generally more active after transection of the nerve cord, the activity cycle of each unit appears to be quite independent of the others. Efferent activity before and after cord transection was recorded simultaneously in the phallic nerve and nerve IX. It can be seen (Fig. 5) that there is no interaction between fibres in the two nerves, their spikes being quite independent, yet the activity in both has been increased by the cord transection. In Fig. 5 (c) a volley in IX coincides with a relatively quiet period in the phallic nerve.

The inhibitory effect

Regions of the nervous system responsible for the inhibitory effect were located in the following manner. Removal of the compound eyes, ocelli, and antennae of the cockroach caused no permanent increase in the efferent output of the last abdominal ganglion. Removal of the brain (supra-oesophageal ganglion) was followed by an increase in small spike activity in 4 out of 8 preparations. Decapitation (or transection of the cercival connectives) was followed by a much more pronounced increase that included volleying in the large fibres. The effect was similar to that caused by transection of the abdominal nerve cord. This shows that, as in the mantis, the sub-oesophageal ganglion is the main source of the inhibitory effect. In some cases transection of the abdominal cord subsequent to

decapitation brought about a small additional increase in the efferent activity, suggesting that the thoracic ganglia may also contribute in smaller measure to the inhibitory effect.

Assuming that suppression of this efferent activity in the intact nervous-system is due to a tonic discharge of inhibitory impulses in descending nerve fibres, it is somewhat surprising to encounter a delay of as much as 15 min between removal of the inhibitory effect by cord transection and the onset of efferent activity. Efforts were made to find out whether the duration of this delay is determined by the site at which the inhibitory pathways are interrupted. There was no evidence that the delay differed when transection of the cord was made in the neck as compared with the abdominal region. However, since the delay was so variable, ranging from a few seconds to as much as 15 min, its duration in relation to the site of transection could have been obscured in the small number of observations that have been made.

The method of cord transection permitted only one release of endogenous activity per preparation, so attempts were made to establish a reversible block of the inhibitory effect. An electronic method was first tried. The passage of direct current through the nerve cord via an anode placed on the cord pitted against an indifferent cathode brought about a small increase in the efferent activity in nerves IX and X in 3 out of 11 cases, but this action was not sustained for the duration of the polarizing current. In the other cases there was no change in efferent activity. In 6 of the 11 cases the nerve cord was transected subsequent to treatment with the polarizing current and in each of these there was the usual increase in efferent activity.

Localized ionic block of descending inhibitory pathways in the nerve cord was attempted through the application of isotonic potassium chloride and sodium-free (sucrose substituted) Hoyle's saline. Isotonic potassium chloride applied locally to the intact nerve cord takes about 30 min to block conduction in the ascending giant fibres, but if the sheath surrounding the cord is removed before potassium chloride application the block occurs in a minute or so (TWAROG and ROEDER, 1956). These agents were applied to intact and desheathed portions of the abdominal nerve cord while monitoring the output of efferent activity in nerves from the last abdominal ganglion. Difficulties were encountered in restricting the potassium chloride to a limited region of the nerve cord, and attempts were made to prevent it from reaching the last abdominal ganglion by submerging the latter in mineral oil. Of 7 experiments in which efferent activity continued in the last abdominal ganglion throughout the rather involved procedure 1 showed no change, 4 showed a slight increase not comparable with that achieved by cord transection, and in 2 there was an immediate increase followed by cessation of all efferent activity. In the latter activity was restored after 5 min by washing with saline, and the cessation could have been due to leakage of potassium chloride to the region of the ganglion.

The failure of these two attempts to block the inhibitory action caused some surprise, and is interesting in itself. Three possible explanations come to mind. First, the methods were technically inadequate. However, the same method of

producing a potassium chloride block was successful with the giant fibres (TWAROG and ROEDER, 1956). Second, the inhibitory fibres have a small diameter and are in some way protected from agents that block the giant fibres. Third, the inhibitory action of the higher centres is exerted by agents other than nerve impulses. The last two possibilities remain entirely open, and the mode of inhibition will be considered further in the discussion.

Endogenous activity in the metathoracic ganglion

Spontaneous nerve activity in the thoracic ganglia of insects was first observed by ADRIAN (1930) and subsequently studied by many workers. The usual method has been to suspend the thoracic chain between two electrodes, suggesting that much of the activity recorded in previous studies occurs in internuncial fibres connecting the ganglia. Electrodes placed below nerves IIIa or V of the metathoracic ganglion revealed that there is also a spontaneous discharge of impulses in efferent fibres from the neurally isolated ganglion (WEIANT, 1958). A series of transection experiments similar to those carried out on the last abdominal ganglion showed that transection of the cervical connectives or decapitation is followed by an increase in the efferent discharge. This took the form of increased frequency of firing in fibres active before the transection and the onset of regular firing in fibres that had been previously inactive.

VOL. 4 1960

As in the case of the last abdominal ganglion, the descending inhibitory effect in either cervical connective acts on both sides of the thoracic ganglia. Transection of one cervical connective was followed by an increase in the efferent activity of nerves on both sides; subsequent transection of the other cervical connective was followed by an additional bilateral increase. As before, the increase was not immediate, but took a minute or more to reach a steady level at which it continued more or less indefinitely. Removal of the sheath (neural lamella) had no significant effect on the efferent activity of a ganglion bathed in Hoyle's saline. However, bisection of the desheathed ganglion in the mid-line was followed by a still greater efferent discharge, sometimes punctuated by bursts, in the nerves arising from both halves. Such activity continued in half ganglia for an hour or more. Other details have been reported previously (WEIANT, 1958).

The action of pharmacological agents

Attempts were made to study the action of various drugs both on the spontaneously active efferents and on the inhibitory system. The technical difficulty of confining the action of a particular substance to one system or the other within the narrow confines of a cockroach made most of the results unmeaningful, and they will not be reported in detail. In general the pharmacological response pattern of the efferent fibres in the last abdominal ganglion was similar to that previously found for giant fibre synapses (TWAROG and ROEDER, 1957) although the threshold concentrations (concentrations producing a detectable change in spontaneous activity) were some 10-fold lower.

THE BEHAVIOUR OF DECAPITATED COCKROACHES

Endogenous efferent activity from the last abdominal ganglion is similarly controlled by sub-oesophageal inhibitory centres in mantis and cockroach. The hazards of courtship caused by the cannibalistic tendency of the female mantis seem to justify the adaptive value of this arrangement in the male mantis. The behavioural significance of a similar neural arrangement in the male cockroach was examined.

ROTH has published extensive descriptions (1952, 1954) of the courtship behaviour of *Periplaneta americana* and other cockroach species. An odour released by virgin females is detected by the male antennae and releases the courting behaviour. This consists of rapid exploratory locomotion, antennal and labial exploration of other cockroaches, wing fluttering, elongation and depression of the abdomen, and elevation of the abdominal tip. WHARTON (1953) has shown that the intensity of these signs of sexual excitation depends on the intensity of the female odour and also upon the density of the roach population. Mutual excitation appears to be essential for full development of the sexual response, isolated males showing little or no response to the female odour. These observations were confirmed. The courtship pattern is very different from that of the mantis, and, as might be expected from the non-predaceous habits of the cockroach, cannibalism plays no part in the interaction of male and female.

Decapitated male cockroaches placed in contact with females never completed copulation and failed to show sexual behaviour that was sufficiently co-ordinated to suggest that this might be possible. However, the posture and movements of various parts of the body did suggest portions of the copulatory action pattern. Unlike the decapitated male mantis the decapitated male cockroach shows no phasic locomotor activity except for brief evasion responses when the cerci are stimulated. When compared with the posture of an intact insect it stands higher off the substrate indicating an increased extensor tonus of the leg muscles. A similar posture is seen in sexually excited intact males and could be ascribed to the increased output of efferent impulses from the thoracic ganglia.

The abdomen of a decapitated insect makes various movements that continue for several hours. These include lowering of the abdomen, slight peristaltic movements of the terminal abdominal segments, bending of the abdominal tip both upwards and to the side, twitching of the cerci, and movements of the phallomeres. Such movements do not occur in intact sexually unexcited males, although they are similar to some of the abdominal movements made during sexual excitation as well as to the abdominal movements made by a decapitated male mantis. However, they never led to effective copulation and they gave the impression of being fragmented and uncoordinated portions of the sexual action pattern. Our observations did not suggest that these movements had any adaptive behavioural significance.

As mentioned above, the stiff-legged posture of a decapitated insect suggests an increase in muscle tonus. Attempts were made to correlate the increased efferent activity in nerve IIIa after isolation of the metathoracic ganglion with changes in muscle tension. There was little or no evidence of muscle potentials

corresponding to the efferent spikes, and it seems probable that most of the released efferent activity was taking place in 'slow' fibres (HOYLE, 1955). These could well be responsible for the tonic postural change seen in the decapitated insect. Of course, it is still possible to evoke large muscle spikes and accompanying quick twitches in the leg muscles by reflex means such as stimulating the cercal nerves (ROEDER, 1948). Phasic movements of the legs occur in decapitated insects, particularly during the grooming of the cerci with the metathoracic leg.

DISCUSSION

A comparison of the physiological experiments on the mantis and roach suggest a very similar neural arrangement. Endogenous and often highly rhythmic discharges occur independently in various efferent fibres of the isolated last abdominal ganglion. When the nervous system is intact this activity is largely or completely suppressed by inhibitory centres located mainly in the sub-oesophageal ganglion. This efferent activity appears to be the source of movements of the abdomen, cerci, and phallomeres in the decapitated insect.

VOL. 4 1960

The adaptive value of this arrangement is clear in the male mantis. Decapitation by the female mantis during courtship releases continuous copulatory movements in the male. These are of greater intensity than similar movements made by intact sexually excited males, and copulation by decapitated males is quite common. Therefore, the co-ordinated copulatory movements correspond to the increased spontaneous efferent activity.

By comparison the sexual behaviour of a decapitated male cockroach is disorganized and fragmentary and does not enable copulation in spite of the apparently similar neural arrangement in the two insects. This suggests that tonic inhibition by the sub-oesophageal ganglion of endogenous efferent activity in the last abdominal ganglion may be a fairly general neural arrangement in insects that has attained full behavioural expression in mantids because of the cannibalistic tendencies of this group. Indeed, although most of the neurophysiological experiments with cockroaches were carried out on adult males, a similar neural arrangement was found in females and nymphs, and was found to extend to efferent nerve fibres in the thoracic ganglia as well as those in the last abdominal ganglion.

This concept of local efferent spontaneity patterned into behaviour through differential inhibition from higher nerve centres differs from the generally accepted notion that local reflex arcs are brought into continued activity through excitation entering them from higher centres. However, it provides an explanation for other observations relating to the operation of the insect nervous system. It was necessary to postulate the interaction of various inhibitory centres in order to explain the control of locomotion in the mantis (ROEDER, 1937) although no electrophysiological observations have been made. The isolated abdomen of the male cecropia moth makes vigorous and continuous copulatory movements and may even eject two or three spermatophores (WILLIAMS, personal communication).

The oviposition by an isolated female abdomen observed in a number of insects (silkworm, KELLOGG, 1970; *Mantis*, CHOPARD, 1914) probably depends

upon a mechanism similar to that found in the males. This arrangement seems to have some species survival value in insects likely to be attacked by predators. Spontaneous nerve activity in isolated insect ganglia has been recorded many times, although there has been no previous study of the mechanism by which it is regulated.

This generalization clearly requires substantiation, but the presence of such a system in two fairly primitive insect species and its obvious adaptive value in one of these prompts some speculation as to its possible phylogenetic origin. Increasing cephalization marks the evolution of arthropod nervous systems from their annelid-like ancestors, suggesting that segmental autonomy is a primitive characteristic. In the coelenterates (PANTIN, 1950) and annelids (WELLS, 1950) rhythmic muscular activity is characteristic both of whole animals and of isolated groups of segments. Perhaps local segmental rhythms are the primitive mode, and one functional trend in arthropod cephalization has been to bring these local rhythms into co-ordination and control by the development of descending inhibitory pathways. If this is so, then inactivity might be considered to be a later acquisition in evolution. The regular rhythmic discharge of motor impulses from the isolated last abdominal ganglion of the roach (Fig. 6 c) bears a suggestive similarity to the patterns of rhythmic movement recorded from annelids (WELLS, 1950).

At first sight it may seem hard to rationalize the evolution and persistence of a spontaneous efferent system. In constructing a model it would seem *a priori* simpler to design a stimulus-response mechanism to accomplish the same end. However, a spontaneously active system that is regulated through inhibition has some of the advantages of a fail-safe system in engineering. The 'safe' in 'fail-safe' must be equated with adaptive value, meaning an attribute that promotes racial survival. Should the input fail or the system be shorn of its input mechanism (as in the male mantis) the action resulting from the released spontaneous activity can only improve the chances of reaching a region of input or of racial survival. Removal or reduction of the input in a system of the stimulus-response type places it in the cul-de-sac of inactivity from which there would seem to be no escape.

The nature of the inhibitory system remains unclear, but this must be attributed largely to the crudity of the methods now available for revealing it. If inhibition plays a part in the patterning of insect behaviour one would expect there to be a number of parallel inhibitory pathways in the nerve cord associated with different behavioural patterns. Transection of the nerve cord must inevitably break all these pathways at the same instant thereby obscuring all differential inhibitory effects. Similarly, the electrophysiological method yields information on nerve activity only at one point (per amplifying channel), and the experimenter is in the position of one wielding an axe while looking through a pin-hole. It is fortunate that the somewhat similar operative technique used by the female mantis in her attack on the male has led, through the action of sexual selection, to the accentuation of one of these behavioural patterns and the inhibitory system controlling it.

The most important general conclusion seems to be that in insects inhibitory control of local endogenous efferent activity by other nerve centres is the rule rather than the exception. Thoracic and abdominal ganglia seem to be generally under the control of the sub-oesophageal ganglion. At the same time, the thoracic ganglia exert some mutual inhibitory effect on each other, and an internal contralateral inhibitory effect on right and left efferent nerves. There is behavioural evidence of inhibitory interactions between the brain and sub-oesophageal ganglion (ROEDER, 1937).

The inhibitory effects at the lower levels reported in the present paper appear to be tonic or continuous, although a temporary waning of inhibition is suggested in Fig. 6 (a). They are not altered by removal of the major sense organs which suggests that the inhibitory systems, like the efferents they control, are in a state of spontaneous activity. Electrodes placed on the connectives of the ventral nerve cord invariably register spontaneous activity in internuncial fibres, but it has not been possible to identify fibres showing this activity with the inhibitory effects. It seems likely that they are relatively small fibres in which impulses are not readily detected. Failure to produce electrotonic or ionic block of the inhibitory effects suggests that the inhibitory pathways differ in several respects from the well-known and readily studied giant fibres.

VOL. 4 1960

Another characteristic not encountered in better known synaptic systems is the delay of as much as 15 min between removal of the inhibitory source and full release of the efferent neurons from the inhibitory action. Assuming that the inhibitory action is mediated by small internuncial nerve fibres, this delay is hard to account for except in terms of a mediator acting in a more diffuse manner and over distances greater than those found at the usual synapse. In other words, the mode of release and action of the postulated inhibitory substance would be intermediate between that of a specific synaptic mediator and a more generally distributed hormone.

In summary, these experiments demonstrate a wide-spread system of spontaneously active efferent neurons controlled by inhibitory systems of various types. The behavioural significance of this arrangement has been demonstrated in the praying mantis, and it must be taken into account in neurophysiological analyses of behaviour in other insects.

REFERENCES

ADRIAN E. D. (1930) The activity of the nervous system of the caterpillar. *J. Physiol.* **70**, 34–35.

CHOPARD L. (1914) Sur la vitalité de *Mantis religiosa* L.; ponte après décapitation. *Bull. Soc. ent. Fr.* **19**, 481.

CRANE J. (1952) A comparative study of innate defensive behaviour in Trinidad mantids. *Zoologica, N.Y.* **37**, 259–293.

EIBL-EIBESFELDT I. and KRAMER S. (1958) Ethology, the comparative study of animal behaviour. *Quart. Rev. Biol.* **33**, 181–211.

HOYLE G. (1953) Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–135.

HOYLE G. (1955) Neuromuscular mechanisms of a locust skeletal muscle. *Proc. roy. Soc.* (B) **143**, 343–367.

KELLOG V. (1907) Some silkworm reflexes. *Biol. Bull., Woods Hole* **12**, 152–154.

LORENZ K. Z. (1950) A comparative method in studying innate behaviour patterns. *Symp. Soc. exp. Biol.* **4**, 221–268.

PANTIN C. F. A. (1950) Behaviour patterns in lower invertebrates. *Symp. Soc. exp. Biol.* **4**, 175–195.

RILLING S., ROEDER K. D., and MITTELSTAEDT H. (1959) Prey recognition in the praying mantis. *Behaviour* **14**, 164–184.

ROEDER K. D. (1935) An experimental analysis of the sexual behaviour of the praying mantis. *Biol. Bull., Woods Hole* **69**, 203–220.

ROEDER K. D. (1937) The control of tonus and locomotor activity in the praying mantis, *Mantis religiosa* L. *J. exp. Zool.* **76**, 353–374.

ROEDER K. D. (1948) Organization of the ascending giant fibre system in the cockroach, *Periplaneta americana*. *J. exp. Zool.* **108**, 243–262.

ROEDER K. D. (1955) Spontaneous activity and behaviour. *Sci. Mon., N.Y.* **80**, 363–370.

ROTH L. M. (1952) A study of cockroach behaviour. *Amer. Midl. Nat.* **47**, 66–129.

ROTH L. M. and WILLIS E. R. (1954) The reproduction of cockroaches. *Smithson misc. Coll.* **122**, (12), 1–49.

TINBERGEN N. (1951) *The Study of Instinct*. Oxford University Press, London.

TWAROG B. M. and ROEDER K. D. (1956) Properties of the connective tissue sheath of the cockroach abdominal nerve cord. *Biol. Bull., Woods Hole* **111**, 278–286.

TWAROG B. M. and ROEDER K. D. (1957) Pharmacological observations on the desheathed last abdominal ganglion of the cockroach. *Ann. ent. Soc. Amer.* **50**, 231–237.

WEIANT E. A. (1958) Control of spontaneous efferent activity in certain efferent nerve fibres from the metathoracic ganglion of the cockroach. *Proc. 10th int. Congr. Ent.* **2**, 81–82 (1956).

WELLS G. P. (1950) Spontaneous activity cycles in polychaete worms. *Symp. Soc. exp. Biol.* **4**, 127–142.

WHARTON D. R. A., MILLER G. L., and WHARTON M. L. (1954) The odourous attractant of the American cockroach, *Periplaneta americana*. *J. gen. Physiol.* **37**, 461–469.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 63 *to* 79. *Pergamon Press Ltd., London. Printed in Great Britain*

THE ACTION OF CARBON DIOXIDE GAS ON AN INSECT SPIRACULAR MUSCLE

G. HOYLE

Zoology Department, University of Glasgow, Scotland

(*Received* 10 *April* 1959)

Abstract—Carbon dioxide gas in low concentrations causes the relaxation of the closer muscle of the spiracle of *Schistocerca gregaria* in the face of continued motor nervous excitation. The gas acts directly on events in the neuromuscular transmission sequence. It causes the magnitudes of the junctional potentials and of the twitch tension to fall, the extent of the fall increasing as the carbon dioxide concentration increases. It was not found possible to relate the change in tension exactly to the change in electrical potential.

VOL.
4
1960

INTRODUCTION

CARBON dioxide gas in concentration in air greater than 1–5 per cent (depending on the species) causes the spiracular valves of insects to open (HAZELHOFF, 1927). In some species they may close again after a while, even when the high concentration of carbon dioxide is maintained. In others, they remain open just as long as the carbon dioxide is present. Primarily the gas acts peripherally but it also acts centrally if the concentration in the haemolymph rises, when it affects the respiratory centres (HAZELHOFF, 1927; STAHN, 1929). The mechanism of the former, peripheral action, is on individual spiracles. It was considered by these two authors to be due to stimulation by the gas of sensory receptors in the thoracic or tracheal walls close to the particular spiracle (*Periplaneta*, HAZELHOFF; *Dixippus*, STAHN). The resulting nervous discharge was supposed to cause a reflex inhibitory action, although its pathway was not specified. CASE (1956) proposed a similar set of carbon dioxide-sensing elements for *Musca*, but more recently (CASE, 1957) in a detailed investigation of the spiracular function in *Periplaneta* he threw doubt on his own and HAZELHOFF's interpretations. He was nevertheless unable to offer any alternative explanation of the mode of action of the gas.

A new aspect on the problem was raised by the work of BECKEL (1956) and BECKEL and SCHNEIDERMAN (1957). They made an isolated preparation of the spiracular apparatus of *Hyalophora cecropia* and found that the valves remained closed. The closure was sustained by a continuous stream of excitation, for when the electrical activity of the muscle was recorded, action potentials were detected, occurring at a regular rate of 12–15 per sec. The nature of the potentials, although recorded with an intracellular electrode from muscle fibres, was not described. When a stream of carbon dioxide was directed at the isolated spiracular apparatus, bathed in saline, the valves opened. BECKEL and SCHNEIDERMAN thought that the spontaneous activity and action of carbon dioxide must be due to the presence of a

local (peripheral) reflex nervous system capable of spontaneous discharge and inhibited by carbon dioxide. But in 200 methylene blue and silver-stained preparations they failed to detect any nerve cell bodies near the muscle.

An alternative explanation, and one which would account for anomalies in earlier experiments, is that the action of carbon dioxide is a direct one, either on the muscle fibres or on the neuromuscular junctions. If this is true some very interesting consequences ensue. With this possibility in mind, experiments were undertaken to investigate the mode of action of carbon dioxide gas on the second thoracic spiracle of the locust *Schistocerca gregaria*. With this preparation, which has been described in detail (HOYLE, 1959), carbon dioxide and/or other gases or gas mixtures can either be directed at the muscle lying in saline or blown directly into the local tracheal system which supplies the closer muscle. In the latter case the gas concentration in the bathing fluid can be kept very low.

METHODS

Gas mixtures of known composition were made up in a pair of aspirators feeding into one side of a double-barrelled glass pipette which was drawn out to a tip diameter of about 50 μ. A second pair of aspirators was filled with air or with oxygen. Gas would only flow through the tips of the pipette at an appreciable

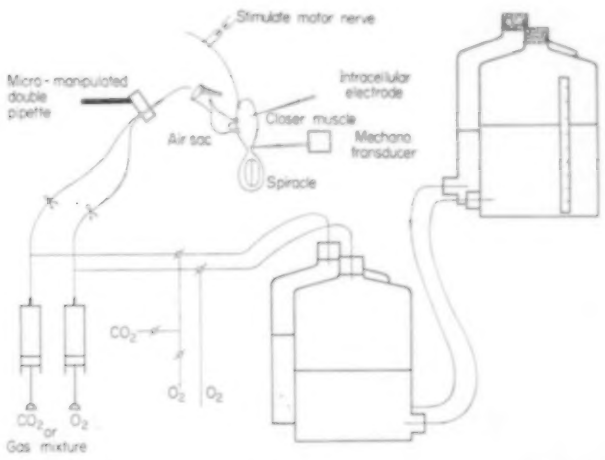


FIG. 1. Diagram of the preparation and device for injecting a 'pulse' of carbon dioxide or gas mixture. Gas mixtures are stored in one pair of aspirators whilst pure oxygen is stored in the other.

rate, as judged by making the emerging gas bubble through saline, under a few atmospheres pressure. To force gas quickly through the pipette, glass syringes were used as the immediate sources by attaching them to the polythene tubing of the supply line by T-junctions and stopcocks. A diagram of the assembly is given in Fig. 1.

The motor nerve supplying the spiracular closing muscle was stimulated by applying brief pulses to it close to the ganglion. Tension of the whole spiracular muscle was recorded with the aid of a finely-pointed needle attached to a crystal gramophone pick-up, the tip of the needle being made to stick in the apodeme. Membrane potentials were recorded from single fibres of the muscle with single or double-barrelled 3 M KCl-filled glass electrodes. The electro-mechanogram and membrane potentials were displayed simultaneously on a cathode-ray oscilloscope and an Ediswan pen oscillograph. All the experiments were carried out at room temperature (20°C—thermostatically controlled room).

RESULTS

Preliminary investigations. Visual inspection of all the spiracles in turn suggested that when a stream of carbon dioxide was directed at a single spiracle, only that spiracle was affected. This is in agreement with all previous investigators. Nothing happens until the valves have opened in the normal sequence of opening and closing movements. Then they may fail to close again, or close only briefly or feebly. Eventually they remain open continually as long as the gas supply is continued. The application of weak mixtures of carbon dioxide (1–5 per cent) was usually not associated with any opening of the valves, but by recording the valve movements with the aid of the piezo-electric crystal, using a high degree of amplification, it could be seen that weak mixtures lower the tension developed in the muscle in response to the stream of nerve impulses in the motor axons which supply it (Fig. 2). Many preparations showed a visible fluttering of the valves, which was also observed by Schneiderman in the *Hyalophora* spiracle. The movements are due to the motor nerve discharging at a frequency which is too low to give a smooth tetanus in the spiracular closing muscle. Each of the small peaks of tension which cause the appearance of flutter is lessened in height by the action of the gas. But the gas, even in pure form applied for a long time, never reduced the tension completely to zero. Furthermore, the rhythm of opening and closing could still be detected in the highly-amplified record of the movements of the valves when the spiracle was apparently wide open under the influence of carbon dioxide. The rhythm is not affected in any way by the gas as long as it is applied only locally, and air is freely supplied to the rest of the animal; the durations of opening and closing continue as before.

To test the site of action of carbon dioxide the main tracheal trunk leading to the spiracle was occluded by inserting a drop of saline through the open spiracle. The gas now had no effect, but when the gas pipette was thrust through the drop and a small volume of carbon dioxide was introduced into the tracheal trunk the valves opened almost instantaneously. As STAHN argued, the gas must either act on receptors inside the tracheal trunk or else it must act in the muscle itself.

The concept of a peripherally-located nervous network would be strengthened if the muscle remained contracted after the nervous connexion of the closing muscle to the ganglion had been severed. But in *Schistocerca* it always relaxed fully after destroying the nerve. There is a possibility that if the muscle is kept in

VOL. 4 1960

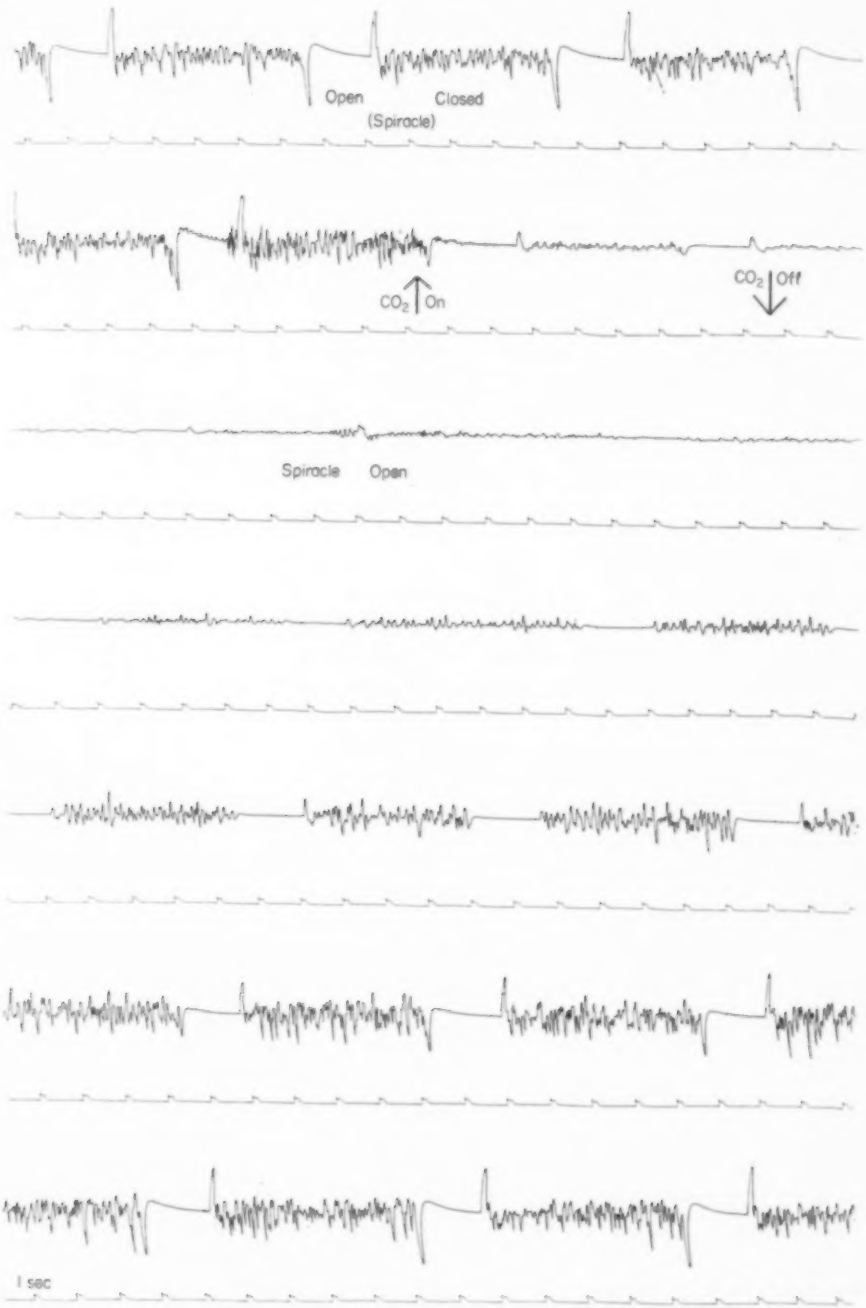


FIG. 2. Action of carbon dioxide on tension during natural respiratory rhythm. Record taken from lip of posterior valve of second thoracic spiracle of left side. Note that the tension peaks are diminished but not abolished. The rhythm of opening and closing phases was not affected by the gas, which was applied locally by injection into the trachea. Time in seconds.

a denervated state for a long time (more than 24 hr) it does in fact go into 'spontaneous' contractions but the nature of these contractions has not yet been examined.

If the action of the gas is not direct but occurs via sensory receptors there should be an irreducible latency in the response to the gas, depending on the speed of the intermediate events conveying excitation due to the gas to sites where inhibition of the motor discharge occurs. This latency would be least when the gas is applied directly to the receptors. Thus it might be possible to locate the receptors by measuring the latency with the gas pipette in different positions. In order to compare the effects of applying the gas at different sites a method of standardized application of the gas is required.

Application of 'pulses' of carbon dioxide. A technique for the introduction of quantitatively similar amounts of gas in similar times was utilized as follows. A sudden force was applied to the plunger of the syringe containing the pure gas so as to reduce its volume by a standard amount. The gas was maintained under pressure for 1 sec and then the plunger was rapidly withdrawn to its fullest extent, creating a negative pressure at the tip of the pipette. Immediately afterwards, pressure was applied to the plunger of the other syringe, which contained pure oxygen. This oxygen thus 'washes' out the carbon dioxide. The net result is a sudden increase in the local carbon dioxide concentration which is later suddenly reduced. By analogy with a pulse of electric current, such a change in the carbon dioxide concentration may be referred to as a 'pulse' of gas.

VOL. 4 1960

Using these 'pulses' of gas it was determined that the latency is less the nearer the tip of the pipette is brought to the point of entry of the fine tracheae entering the muscle itself. That is, by inserting the gas into the tracheal sac which lies between the main tracheal trunk and the muscle, and bringing the gas to the mouths of the final openings into the muscle, the least latency is obtained. The motor nerve was stimulated at intervals of 1 sec and the twitch of the whole muscle recorded with the aid of a pen oscillograph. A pulse of carbon dioxide was then injected, the moment of injection being recorded on the pen oscillograph. The heights of successive twitches were measured and plotted against time. By drawing a curve between the points and extrapolating (cf. Fig. 6), the time of onset of action of the gas can be estimated very roughly. It must be borne in mind that the gas will not reach all parts of the muscle simultaneously, so if the action is a direct one it will begin to be apparent when those muscle fibres nearest the source of the gas have been affected, and will increase in magnitude progressively as more fibres become involved, in addition perhaps, to an increase of its effect with time in the regions already affected. The results of such experiments show that the latency is either very small indeed or else there is none, other than the time required for the gas to diffuse to the muscle fibres. It must be concluded that there is every reason to suspect that the action of the gas is a direct one.

Action of carbon dioxide on the neuromuscular mechanism. General. In evaluating results of experiments on the action of the gas on neuromuscular transmission it should be recalled that we are studying the action of the gas on whole muscle

tension, which is the sum of the individual tensions of many muscle fibres. The electrical activity is recorded from individual fibres, and it is only by consideration of probability that we can correlate the two events. Also, the muscle-fibre membrane becomes progressively damaged as it is made to contract repeatedly with an electrode inserted, so only a few quantitative comparisons, for example of the response magnitude at different concentrations of carbon dioxide, can be made with the electrode in the same fibre. Fortunately, the electrical responses of different muscle fibres are not greatly dissimilar, being largely in the nature of end-plate potentials. But they differ in regard to the extent to which they give active membrane responses (HOYLE, 1959). The data to be presented have been selected from muscle fibres with a resting potential of not less than 50 mV and an electrical response of between 15 and 40 mV (i.e. largely composed of junctional potential alone).

The lowest concentration of carbon dioxide in oxygen which has just led to a reduction of the twitch tension when applied in the tracheal sac, was in some

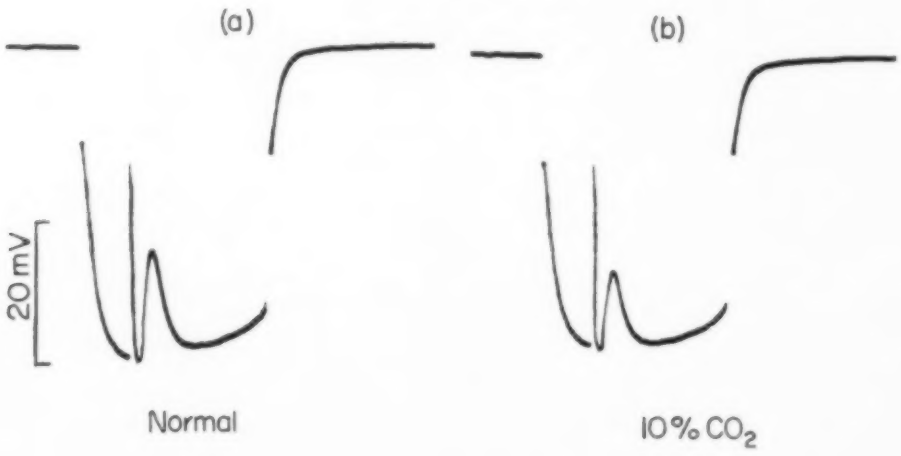


FIG. 3. Action of carbon dioxide on the muscle-fibre membrane resistance and the height of a junctional (end-plate) potential. Constant-current square pulse applied to membrane: (a) in oxygen; (b) in 10 per cent carbon dioxide in oxygen. Note the reduced height of the junctional potential and the smaller magnitude of the applied square pulse (potential recording) in carbon dioxide.

preparations as low as 5 per cent but in others it was slightly higher. The concentration achieved in the muscle by applying the gas in the trachea cannot be accurately gauged. It may be appreciably less than that which is applied in the pipette. Usually 10 per cent carbon dioxide had a considerable effect, reducing the twitch tension to one third normal or even less. It was not considered possible to study reliably the quantitative relationship between carbon dioxide concentration

and tension with the present method of injecting the gas, but it was evident that the tension falls off quickly with increasing concentration, between threshold and 15 per cent carbon dioxide, beyond which the curve flattens (Fig. 4).

Some preparations show an effect during continued application of the gas at the threshold concentration after only 3 sec. Others require up to 10 sec before an effect is apparent. The differences may be due to physiological causes, or they may be due to different degrees of access of the gas to the sites of action.

When the length of an applied 'pulse' of carbon dioxide was reduced progressively so as to apply threshold concentration for shorter and shorter times up to 'utilization' time, it was found that the period during which there is a weaker twitch tension cannot be reduced to less than about 4 sec. This may denote the time required for the carbon dioxide concentration to fall back below threshold, or it may mean that high carbon dioxide leads to a further reaction, with its own time-course.

Effects on electrical potentials. Weak concentrations of the gas, which have a small effect on twitch tension, did not have any significant effect on either the membrane resting or junctional potentials. Even during tension changes down to one half normal it was not possible to detect any change in the potentials in most

VOL. 4 1960

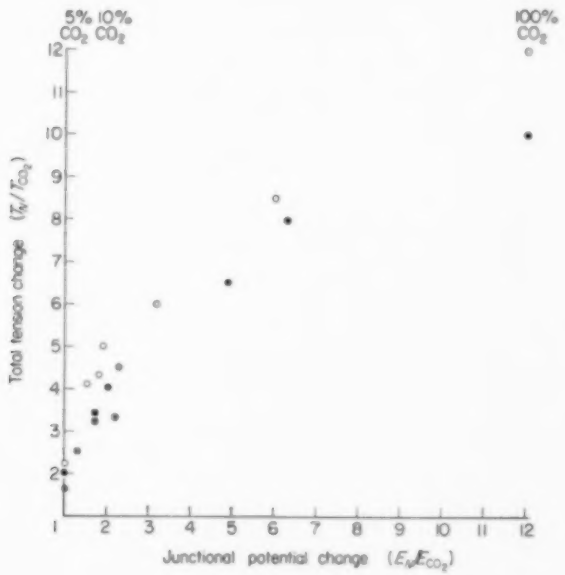


FIG. 4. Fractional change in tension: normal tension/reduced tension in raised CO_2 = T_n/T_{CO_2}; plotted against fractional change in electrical potential (normal electrical potential/reduced electrical potential in CO_2 = E_n/E_{CO_2}). The electrical measurements were taken from four different muscle fibres. Various concentrations of carbon dioxide were used to reduce the tension; electrical potentials and twitch tensions (of whole muscle) were measured when the response had reached a steady state.

of the muscle fibres penetrated. But greater concentrations regularly had a detectable effect. The tension, under the influence of high concentrations of carbon dioxide, fell to as low as 1/15 normal. At the same time there was a reduction in the height of the electrical response which was great in some fibres, small in others. The maximum reduction obtained was to 1/12 normal.

The resting potentials were much less affected than the end-plate potentials. Many fibres showed no effect even when pure carbon dioxide was applied for some minutes. Others showed a small fall in the value of the potentials, i.e. they were slightly depolarized under the action of the gas. In one muscle fibre a depolarization of 8 mV was registered during the application of pure carbon dioxide.

To test the effects of carbon dioxide on the resistance of the membrane, a constant-current rectangular pulse was applied to it through one barrel of a double-barrelled intracellular electrode. The potential change was recorded through the other (tip resistance about 19 MΩ). The magnitude of the recorded pulse depends on the transverse resistance of the membrane, the change in its size during the application of CO_2 (via the tracheal supply) affording a measure of the action of the gas on membrane conductance. An increase in size would indicate lowered membrane conductance (increased transverse electrical resistance). The recorded pulse height actually fell during the application of gas, by from 5 to 10 per cent (Fig. 3). More detailed work is required on this, but the preliminary results show that membrane conductance is increased by the gas. This observed change could involve the whole of the muscle-fibre surface, or it may be confined to the probably rather numerous subsynaptic regions.

In the experiment shown in Fig. 3 the electrical response to stimulation of the nerve was elicited at the same time as a polarizing rectangular pulse was applied to the membrane. The response was a junctional potential (j.p.), and this was timed to arrive at the point of highest membrane potential. If the reduction in the height of the j.p. is solely due to the action of the gas on the membrane permeability the peaks of the potentials should reach the same height (similar equilibrium potential). Under carbon dioxide the potential actually reached a lower height, sometimes very much lower. Hence the gas probably acts not only on muscle membrane, increasing permeability to ions, but also on the mechanism of synaptic transmission, resulting in a reduction of the magnitude of the synaptic reaction.

Correlation of electrical and mechanical events. The question can now be raised whether carbon dioxide causes inhibition of the mechanical response by virtue of its action on the subsynaptic membrane, or on the synaptic reaction, or in some other way. Partial mechanical inhibition of the kind found in the spiracular muscle is analogous to that obtained in crustacean muscles by the action of the inhibitory nerve supply (KUFFLER and KATZ, 1946). In crustacean muscles the relationship of electrical potential to tension is by no means clear. In some of them mechanical inhibition can occur without there being any detectable effect on the magnitude of the excitatory junctional potentials (HOYLE and WIERSMA, 1958). The mechanism of the inhibitory action is not apparent in these cases, and it has been necessary

to propose that the inhibitory transmitter substance has an action on a postulated coupling mechanism situated near the surface of the muscle fibre. This mechanism affords a link between the surface excitatory reaction and the contractile process.

It may be that there is a comparable coupling mechanism in insect muscle and that in the spiracular closing muscle carbon dioxide can act on it. The relationship between the tension and electrical potential magnitude under the influence of various concentrations of carbon dioxide has been studied by plotting the ratio of normal tension to reduced tension against the ratio of the normal electrical response to reduced electrical response. The results were obtained by supplying a continuous stream of the gas in oxygen to the tracheal sac, whilst stimulating the nerve at intervals of about 1 sec. The magnitudes of the electrical responses and

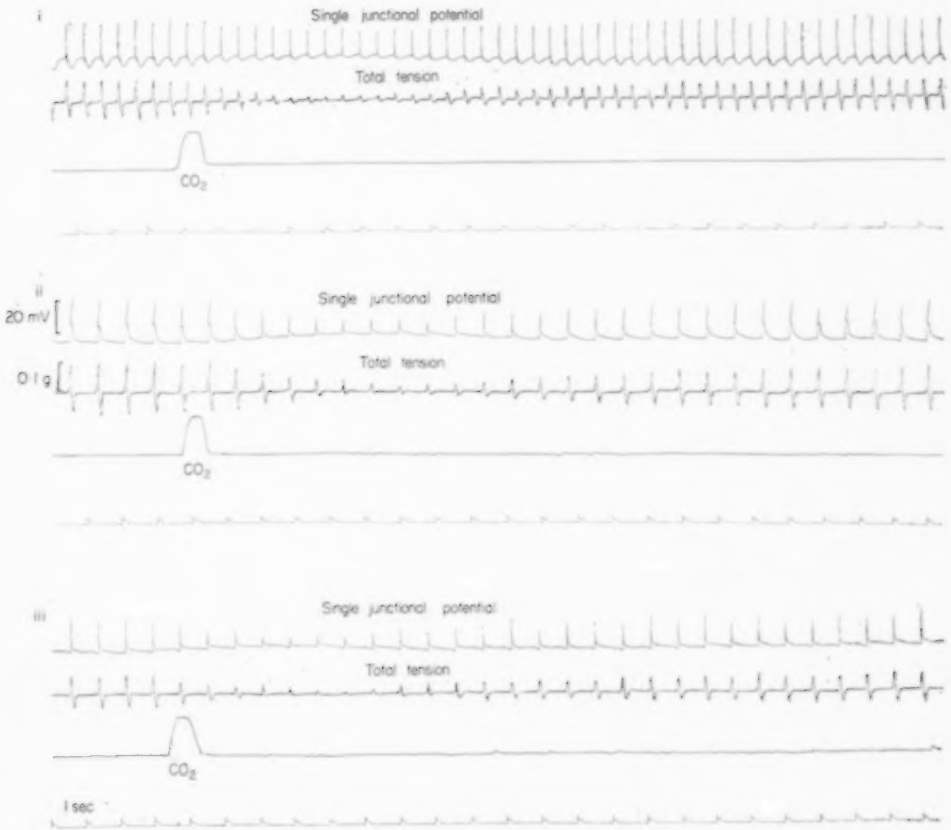


FIG. 5. Action of 'pulse' of carbon dioxide on the electrical and mechanical events associated with spiracular movements. The motor nerve was stimulated at regular intervals and a 'pulse' of carbon dioxide was injected as indicated on the third trace from the top in each record. Selected records from three different preparations. Time in seconds. Calibration given in (ii) applies throughout.

VOL. 4 1960

of the twitch tensions were recorded continually, and the values in the steady state used to determine the ratios. The gas concentration was then increased slightly and the experiment repeated. The results obtained are shown in Fig. 4.

A line drawn through the points crosses the ordinate (ratio of tensions) at a positive value and there are points actually lying on the ordinate. The scatter of the points is rather great, and is such as not to preclude the possibility that the true curve passes through the origin. In searching for a possible explanation of points lying on the ordinate, the minute abortive spike responses of some fibres comes to mind. It may be that at low concentrations of carbon dioxide these responses are affected rather than the junctional potentials. The electrical measurements of Fig. 4 were obtained from muscle fibres giving almost wholly junctional potentials, as far as could be determined. Fibres giving the secondary responses occur sparsely, and it has not yet been possible to test in an adequate manner the possibility of the spikes being the first part to be affected.

It is nevertheless quite certain that the continued application of a low concentration of carbon dioxide produces a relatively large fall in twitch tension which is associated with a very small fall of electrical response, and this throws into relief the problem of the relationship between excitation and contraction, and the mechanism by which they are coupled.

A further study of the relationship between tension and electrical potential was undertaken, using only brief 'pulses' of carbon dioxide to bring about an alteration in the response. Three examples of these experiments are illustrated in Fig. 5. The motor nerve was stimulated at regular intervals and the resulting twitch tensions and electrical potentials recorded. The time-courses of the changes in mechanical responses were similar in all three, following in each experiment the injection of 1 sec pulses of pure carbon dioxide washed out by pure oxygen. The peak effects were reached in 4, 5½, and 7 sec respectively, and full recovery took 14–16 sec in each. The electrical results were more variable. In (i) the maximum reduction in junctional potential was only to 2/3 normal and the depolarization was only 2 mV. In (ii) and (iii) the reductions were to 1/3·6 normal and 1/4·3 normal with depolarizations of 6 mV and 4 mV respectively. In interpreting the results we encounter the difficulty referred to earlier, for it cannot be argued that the differences must represent genuine physiological variations, since they could have been the result of different extents of access of the gas to different fibres.

An apparent correlation between the electrical and mechanical events is clearly evident in the records, particularly those of (ii) and (iii), which are plotted graphically in Fig. 6.

A consequence of the hypothesis that the magnitude of the isometric twitch tension is determined by the magnitude of the electrical response is that during recovery from the action of carbon dioxide the curve tension against electrical response should retrace the steps which it followed during the onset of action of the gas. Several experiments have been made in which the twitch tensions and junctional potentials were recorded at 1 sec intervals during the declining phase

under pure carbon dioxide and during recovery in oxygen. Four examples are plotted in Fig. 7. During the declining phase the electrical responses fall in magnitude faster than the tension falls. In order that the curve should retrace its steps during recovery, tension would have to return to normal a little faster than electrical response. In fact this does not happen, the electrical recovery occurring

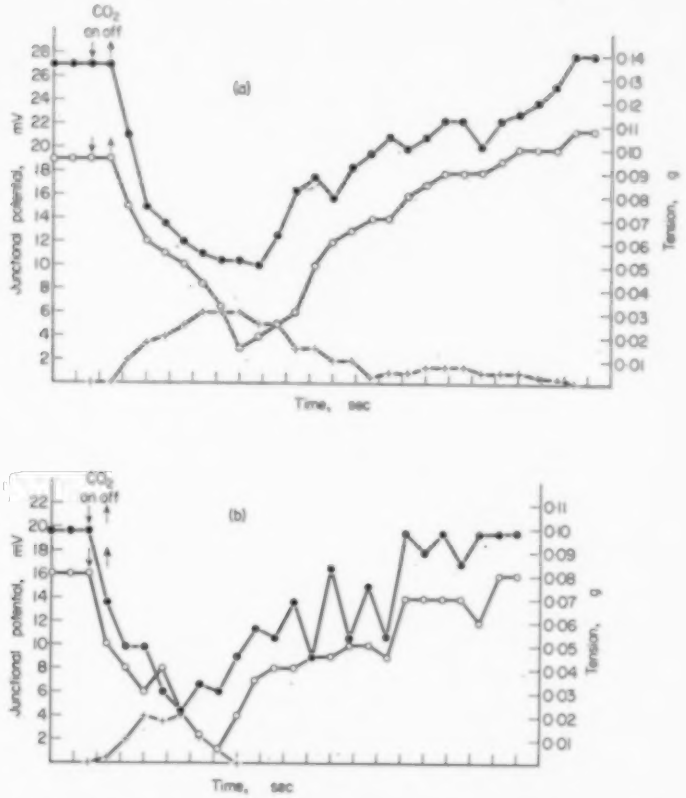


FIG. 6. Correlation of electrical and mechanical events. Action potential from single muscle fibre—upper line, solid points; tension from whole muscle—middle line, open circles; membrane depolarization—lower line, crosses. The scale for the action potentials also applies to the depolarization.

in advance of the mechanical one. This would not present any difficulty in interpretation if the decline and recovery took place quickly, but since the latter takes several seconds it means that during recovery there may be a lag of a second or more between electrical potential recovery and twitch tension recovery. It must be concluded that reduction in tension due to carbon dioxide could be a consequence partly of the attenuation of the action potential but may also be caused in part or wholly by a more direct action.

Following moderately long treatment with carbon dioxide, the twitch tension may continue to increase during recovery after it has returned to normal, showing a supernormal phase which is maintained for up to a minute.

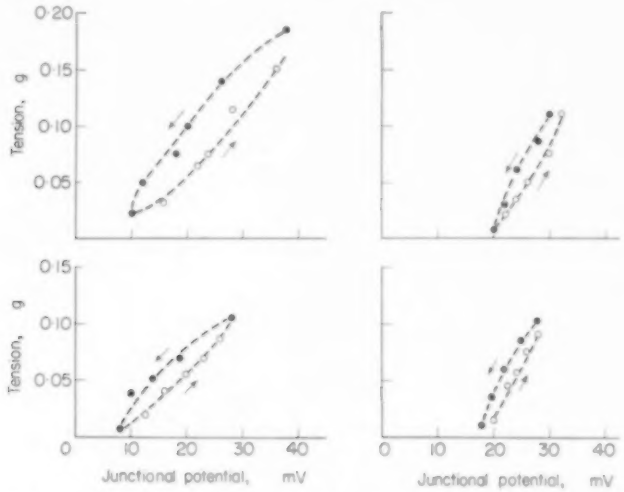


FIG. 7. Total tension plotted against electrical response of a single muscle fibre during the onset of action of carbon dioxide (solid circles) and recovery from it (open circles). Four preparations.

The action of oxygen. BECKEL and SCHNEIDERMAN found that oxygen reduced the sensitivity to carbon dioxide in their preparations, i.e. a higher concentration of carbon dioxide was needed to cause relaxation in the face of the spontaneous excitatory rhythm in an oxygen-rich medium. The explanation may be that oxygen leads to a higher twitch tension in the moth. In the present experiments a few, though not the majority, of muscles gave enhanced twitch tension following injection of pure oxygen (after air) into the trachea. The effect was not more than about a 15 per cent enhancement, and the concentration of carbon dioxide which was then required to cause a reduction was not greater than normal.

The action of carbon dioxide during the normal respiratory rhythm. The action of carbon dioxide on the electrical and mechanical events associated with the rhythmic discharge of the mesothoracic ganglion were investigated by leaving the spiracular nerve intact. The gas caused a fall in the strength of the closing movements, as described earlier, but it did not affect in any way the pattern of the discharge of the nerve as evidenced in the electrical potentials recorded from muscle fibres. In the higher concentrations the magnitude of the electrical responses was of course reduced (Fig. 8). The time-course of its action closely resembled that obtained when stimulating the nerve electrically.

Action of carbon dioxide introduced into the bathing fluid. Pure carbon dioxide gas was bubbled into the small volume of saline surrounding the closer muscle

and the effect on electrical and mechanical activity recorded. Most of the muscles examined failed to show a reduced twitch tension even when the carbon dioxide was bubbled for a long period. A few did show effects similar to those obtained by injecting a low carbon dioxide concentration into the tracheal system, but the response only appeared after prolonged bubbling. The saline (HOYLE, 1953) and

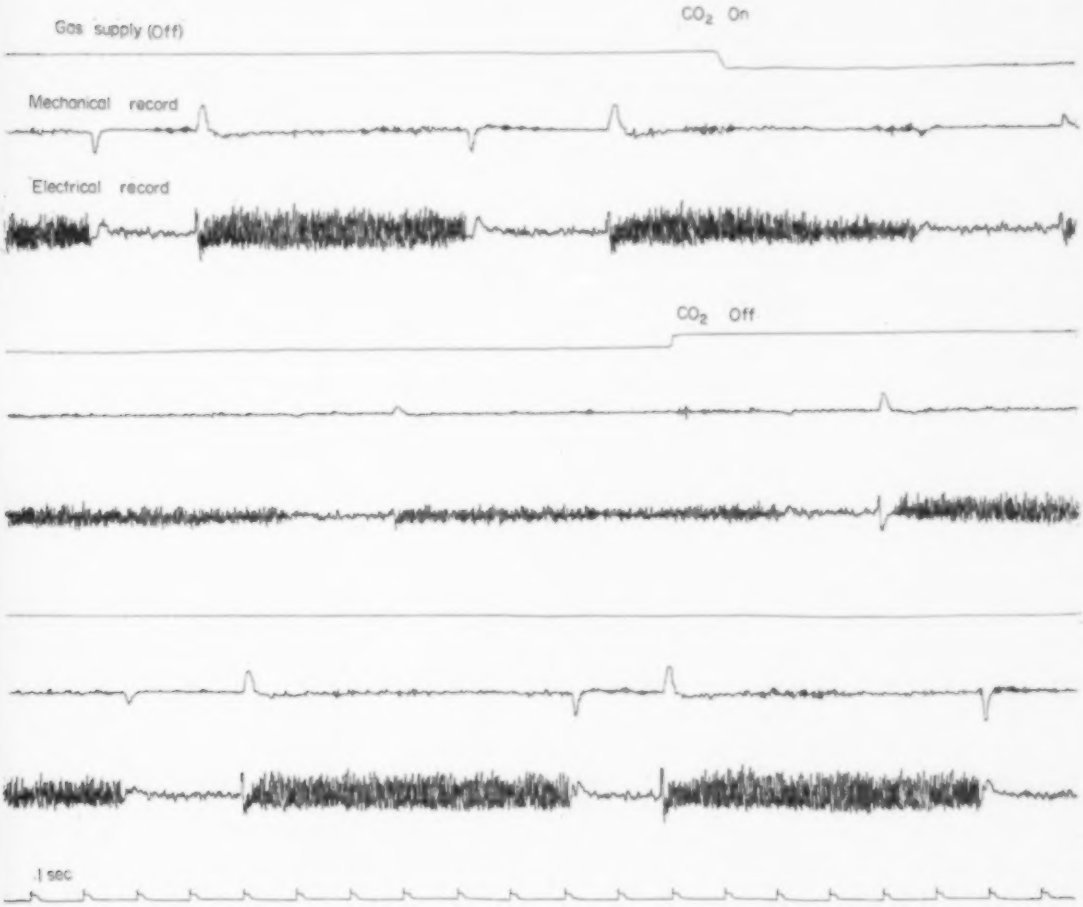


FIG. 8. Effect of carbon dioxide on normal rhythm. Continuous record of electromechanogram (middle line—frequency of discharge high giving nearly complete tetanus) and action potentials recorded from a single muscle fibre. Tension and action potentials are both markedly reduced but the rhythm of opening and closing is unaffected by the locally applied gas.

also the tissues have some buffering capacity, so the gas would be partially absorbed. But it seems probable that the gas is failing to pass through to the sites of action. Saturated solutions of carbon dioxide in sodium chloride also failed to

VOL. 4 1960

cause any immediate reduction of the twitch tension. It is evident that it is the partial pressure of carbon dioxide gas in the tracheae which is the significant factor in causing an interference with neuromuscular transmission.

Action of other gases. In order to test the possibility that carbon dioxide acts by virtue of its acidity, a possibility which was rejected by BECKEL and SCHNEIDERMAN, pure hydrochloric acid gas and pure ammonia gas were injected in turn into the tracheae. This is drastic treatment, but neither gas had any effect within several minutes, after which there was irreversible deterioration of the twitch. Thus it is unlikely that an altered pH is the cause of the response: carbon dioxide is exerting a specific action.

DISCUSSION

The foregoing experiments enable us to conclude that the control mechanism of the locust second thoracic spiracle does not involve a local (peripheral) nervous system of specialized type having carbon dioxide-sensitive receptors. Nor is the central nervous system involved in the apparently reflex inhibition of the closure by carbon dioxide gas. Its action can be interpreted as a direct one on the processes involved in the transmission of excitation at the neuromuscular junctions.

In the previous paper (HOYLE, 1959) it was shown that the membrane of the muscle fibres is only very weakly electrically excitable. The electrical responses to nervous excitation consist almost entirely of the local short-circuits occurring in the neuromuscular junctional regions following the arrival of the transmitter substance, namely the end-plate potentials. The electrically excitable abortive spikes arising in the membrane as a result of the current flow of the e.p.p. constitute only a small fraction of the total response. What the significance of the tiny active membrane responses might be in initiating contraction we cannot say, for indeed, we cannot yet answer the question: what are the significant events in the transmission process for initiating contraction?

Whatever determines the strength of the contraction of the closer muscle, it is certainly related in part to the events which determine the size of the electrical potential. The smaller junctional potentials associated with the slow axon give rise to smaller twitch contractions than the fast (HOYLE, 1959). Carbon dioxide lowers the height of the junctional potential. It may do this in one of two ways: (a) by reducing the amount of transmitter substance liberated at the nerve endings, or (b) by reducing the sensitivity of the postsynaptic membrane to the action of the transmitter. There is a great deal of evidence to show that in vertebrates some neuromuscular blocking agents, in particular curare, act by competing with the receptor molecules in the subsynaptic membrane, rather than by reducing the quantity of transmitter substance. Since carbon dioxide causes increased conductance of the muscle-fibre membrane, leading in some cases to a depolarization, it is certain that it can affect the properties of the membrane. Hence it is possible that carbon dioxide acts in a manner resembling that of curare in vertebrate muscle.

The gas also parallels in some ways the action of the inhibitory substances of crustaceans, as mentioned earlier. Both lead to a small increase in membrane

permeability (cf. BOISTEL and FATT, 1958). Also, following moderately prolonged inhibition in crustaceans there are enhanced mechanical and electrical responses to excitation given alone. Following recovery from carbon dioxide there are also sometimes enhanced responses in the spiracular closing muscle. There is an even more striking parallel in the fact that at low levels of mechanical inhibition there is no discernible effect on the size of the electrical response in either the crustacean or the insect muscle.

It is in the investigation of the unknown field of coupling of excitation to contraction that the spiracular muscle preparation may prove to be most useful, for it gives us a tool in which twitch tension can be varied over a wide range. It has usually been held in recent years that tension in muscle is directly related to membrane potential, although several recent papers have cast doubt on this interpretation.

The relationship does not hold good for many crustacean muscles. In the spiracular muscle also, two factors argue against such a relationship. Firstly, there is the hysteresis which occurs in the curves of membrane potential against tension under carbon dioxide, and the recovery from it. If electrical potential change directly determines tension, then the steps during recovery should retrace those occurring during decline. In fact they are separated. Secondly, there is the curve relating the ratio of normal to reduced tensions with the corresponding ratio of electrical potentials in the steady-state condition under the influence of carbon dioxide. The initial part of this curve probably starts at a positive value on the ordinate and it rises very steeply indeed. Thus it may be that some factor in the transmission of excitation, other than the electrical potential as such, is responsible for initiating contraction. On this basis contraction could be inhibited without there being any detectable effect on the electrical manifestations of the transmission process.

The transmitter action in all neuromuscular transmission processes is to cause the permeability of the subsynaptic membrane to increase to many ions, thereby giving rise to a junctional potential which is in effect a short-circuit of the rest of the muscle-fibre membrane. This in turn gives rise by purely electrogenic action to an active response of the rest of the membrane if the current flow is large enough and the membrane excitable. In vertebrate skeletal muscle it is the latter process, propagating outwards from the junctional regions, which excites the muscle to contract. In many invertebrate muscles the junctional potentials, which are distributed along the length of the muscle fibre owing to a multiterminal innervation, can themselves bring about muscular contraction. The secondary, active membrane responses involve particularly the movement inwards of sodium ions and outwards of potassium ions, but as the general permeability of the membrane rises during both the junctional (primary) and electrogenically evoked (secondary) potentials, many ions must tend to move across the membrane during their progress. It may be that the movement of minor ions, Ca^{2+} for example, which do not contribute greatly to the potential changes, is the real determinant of coupling, at least in arthropod muscle. The action of carbon dioxide may, in

VOL. 4 1960

part, be due to a direct interference with the balance of these ions. The anatomy of the neuromuscular junction permits the gas to act there directly as well as on the non-junctional muscle-fibre membrane.

The details of the complex mode of action of carbon dioxide in the whole animal remain to be worked out. But it is evident that the direct action of the gas on the neuromuscular junctions provides a form of safety-valve. The centrally-initiated motor nervous discharge looks after the needs of the animal as a whole. Its instructions to a particular spiracle apparently take no cognizance of local conditions there, but if there is a dangerously high accumulation of carbon dioxide in the region of that spiracle then it will open and allow the gas to escape, although all other spiracles may keep closed to prevent desiccation.

SUMMARY

(1) The action of carbon dioxide gas on the functioning of the closer muscle of the second thoracic spiracle of the locust *Schistocerca gregaria* has been investigated. The muscle was excited to contract by electrical stimulation of its motor nerve. Nearly isometric tension was recorded with the aid of a piezo-electric crystal and electrical membrane potential changes were recorded from single muscle fibres with intracellular electrodes.

(2) A method for briefly raising the local carbon dioxide concentration is described; the resulting gas supply is termed a 'pulse' of gas. Pulses were injected directly into the local tracheal supply.

(3) Carbon dioxide acts inside the muscle, interfering with neuromuscular transmission by affecting the subsynaptic membrane.

(4) The conductance of the muscle-fibre membrane is increased by high concentrations of the gas. In some fibres this is associated with a depolarization.

(5) The height of the electrical responses to nervous stimulation is markedly reduced by high concentrations of carbon dioxide.

(6) Low concentrations of carbon dioxide cause the isometric twitch tension to be reduced although there is no appreciable reduction in the height of the electrical responses.

(7) The mode of action of carbon dioxide in reducing the mechanical response may be due in part to a direct action on the mechanism coupling the nervous excitatory response to the contractile elements.

(8) No evidence was found for a peripheral reflex network sensitive to carbon dioxide and affecting spiracular closing. The gas did not affect the motor nervous discharge in any way when it caused opening of the spiracle following local application.

REFERENCES

BECKEL W. E. (1955) The morphology, histology, and physiology of the spiracular regulatory apparatus of *Hyalophora cecropia* (L.) (Lepidoptera). Ph.D. thesis, Cornell University.

BECKEL W. E. and SCHNEIDERMAN H. A. (1957) Insect spiracle as an independent effector. *Science* **126**, 352–353.

BOISTEL J. and FATT P. (1958) Membrane permeability change during inhibitory transmitter action in crustacean muscle. *J. Physiol.* **144**, 176–191.

CASE J. F. (1956) Carbon dioxide and oxygen effects on the spiracles of flies. *Physiol. Zool.* **29**, 163–171.

CASE J. F. (1957) The median nerves and cockroach spiracular function. *J. Ins. Physiol.* **1**, 85–94.

HAZELHOFF E. H. (1927) Die Regulierung der Atmung bei Insekten und Spinnen. *Z. verg. Physiol.* **5**, 179–190.

HOYLE, G. (1953) Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–135.

HOYLE G. (1959) The neuromuscular mechanism of an insect spiracular muscle. *J. Ins. Physiol.* **3**, 378–394.

HOYLE G. and WIERSMA C. A. G. (1958) Inhibition at neuromuscular junctions in crustacea. *J. Physiol.* **143**, 426–440.

KUFFLER S. W. and KATZ B. (1946) Inhibition at the nerve muscle junctions in crustacea. *J. Neurophysiol.* **9**, 337–346.

STAHN I. (1929) Über die Atmungsregulation, besonders die Kohlensäureregulation, bei *Dixippus morosus* und *Aeschna grandis*. *Zool. Jb. allg.* **46**, 1–86.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 80 *to* 91. *Pergamon Press Ltd., London. Printed in Great Britain*

THE STIMULUS TO FEEDING IN LARVAE OF *TENEBRIO MOLITOR* L.

D. R. P. MURRAY

Department of Zoology and Applied Entomology, Imperial College, London, S.W.7

(*Received* 9 *May* 1959)

Abstract—The feeding behaviour of larvae of *Tenebrio molitor* has been studied from the standpoint of the modern concept that phytophagous insects may require a phagostimulant for normal feeding. It is shown that *Tenebrio* larvae discriminate between different parts of the wheat grain, preferring the bran, and that when this is all consumed the total amount eaten falls markedly. Evidence is offered that this is not a nutritional effect but a true response to a feeding stimulus.

Tenebrio larvae will not eat powdered cellulose even when previously starved, but the addition of only 2½ per cent flour causes them to eat the mixture. If the particles are fine enough the larvae cannot avoid eating cellulose in addition to the nutritive constituent. Yet they are still able to exercise some discrimination, for calculations from the increase in true body weight proves that the percentage of flour in the material actually ingested is higher than the percentage in the mixture offered. Complete discrimination can be effected if the stimulant constituent consists of larger particles. The response-promoting substance is localized in the inner layers of the bran. The feeding response is not evoked by simple proteins, carbohydrates or fats. Among natural substances other than wheat flour, very strong responses are evoked by dried yeast and by fishmeal.

THE common mealworm, *Tenebrio molitor* L., is one of the easiest insects to rear in the laboratory, and has been used for research into almost all branches of insect physiology. When used in feeding experiments the assumption has nearly always been tacitly made that the larva of the mealworm will eat anything offered to it, provided that it is presented in powdered form. Although FRAENKEL and BLEWETT (1943) stated of stored products insects in general '. . . the taste and smell of the food is probably of great importance . . . it may well be that an otherwise adequate food is not taken on account of the absence of adequate sensory stimuli', in subsequent work they largely ignored this cautionary proviso and always interpreted failure to grow as an indication of lack of some dietary essential. In the particular case of *Tenebrio*, while announcing (FRAENKEL *et al.*, 1950) that for several years they had failed to grow this insect on a synthetic diet, they never considered the possibility that the larva was not eating. Furthermore, in presenting the animal with a synthetic mixture, the assumption was made that this was uniformly eaten without discrimination of one kind of particle from another.

This outlook is not in accord with the views that have grown up over recent years in regard to phytophagous insects. It is now generally accepted that sensory stimuli play an important part. DETHIER (1953) states that the behaviour pattern

of normal feeding can be broken down into three parts, orientation to food, biting response and continued feeding. The first of these is of primary importance if the larva has to seek food from a distance, and may be ignored in *Tenebrio*. The biting stimulus was at first thought to be the same as the orientation stimulus, particularly in lepidopterous larvae feeding on Cruciferae, where both roles were ascribed to mustard oil, but THORSTEINSON (1953) proved conclusively that it was the odourless glucosides sinigrin and sinalbin, and not the mustard oil liberated from them by enzymic hydrolysis, which constituted the biting stimuli for *Pieris brassicae* and *Plutella maculipennis*. But although leaves of unacceptable plants such as *Pisum sativum* were made acceptable by painting with sinigrin or sinalbin, agar gels containing sinigrin were not eaten unless some nutrient material was also present, suggesting a difference in the requirements for a biting response and continued feeding, though it is not yet clear that this difference is general for all species.

The nomenclature in use in this field of study is not yet standardized, but the terms in current use are summarized by DE WILDE (1958) and THORSTEINSON (1958). Substances which while devoid of nutritional value themselves are nevertheless necessary for the proper feeding response to occur, are variously designated acceptants, attractants, phagostimulants, token stimulants, or secondary plant substances.

VOL. 4 1960

FRAENKEL (1958) now accepts the view that 'secondary plant substances' determine the food specificity of insects which feed on the green parts of plants, but does not consider that the principle extends to grain-eating insects.

The work to be described here sets out to discover to what extent there is discrimination in choice of food by *Tenebrio* larvae, and whether there is required for normal feeding any special stimulant. Proof of discrimination (unless it were caused by particle size alone) would suggest the existence of a taste stimulus; demonstration of substances which did not stimulate eating would confirm it.

VALENTINE (1931) concluded that *Tenebrio* adults locate their food by an olfactory response but the study was not extended to larvae.

MATERIAL

Colonies of *Tenebrio molitor* larvae were obtained by placing 12–18 pupae in a large glass jar (7 lb capacity) containing a shallow layer of a mixture of wholemeal flour and middlings with about 5 per cent dried yeast. Rolls of filter paper were also placed in the jar. The beetles which emerged were generally to be observed walking or resting on these rolls. The jars were kept in a room at 25°C and about 70 per cent relative humidity. In about 8 weeks' time small larvae were seen. The filter-paper rolls and any surviving adult beetles were then removed, together with debris of partly eaten dead adults, and a large supply of food was added. The jars were returned to the constant temperature room and required no further attention. All the larvae in any one jar were thus approximately the same age, and when a sample was required, any which were much below the average size for the colony could be rejected as abnormal. This is a decided advantage. It is perfectly possible to have self-perpetuating colonies with adults, pupae and larvae of all ages existing together, but under such conditions abnormal larvae always accumulate, for the following reason. There are always some larvae which grow very slowly and on reaching a weight of about 20 mg

cease to grow entirely. After remaining this size for a period that may last for months they decline in weight and die. These larvae cannot be distinguished by appearance: they are not sluggish in movement, they are capable of moulting though at less frequent intervals, and they have not been shown to contain more than the usual number of parasitic protozoa. Owing to their long life they are bound to accumulate, and some will be found in every sample of small larvae taken from a self-perpetuating colony. The culture method here employed identifies and eliminates them.

DISCRIMINATION IN CHOICE OF FOOD

A. *Discrimination between different parts of natural wheat grain*

A colony reared as above and 3 months old was found to contain 44 larvae. The diet, it was noted, now consisted wholly of fine material, although coarse middlings had originally been present. The larvae were divided into two groups as alike in size distribution as possible; one group was put back into the existing diet while the other was put into fresh diet. A fortnight later the first group had gained 10 per cent in weight but the second group had gained 23 per cent. In the next fortnight the larvae in the second group began to pupate, and all 22 larvae in this group pupated before any in the first group did so. One possible explanation might be that the larvae excreted into the diet some substance either toxic to further development or repellent to further eating. But the fact that the coarse constituent of the diet had disappeared suggests the alternative view that there had been discrimination, that the preferred material had been removed, and that for lack of this constituent the larvae were eating less. The first view was in fact eliminated by showing that a new addition of middlings to the old diet was all that was required to make it as effective as a fresh sample.

The amount of frass passed on various diets differs. Frass was estimated in the following way. The mixture of frass and food was poured on to a sheet of paper which was then held in one hand and tapped underneath with the other hand. If the paper was tilted the frass rolled out on to a clean sheet. 'Tappings' consisted wholly of frass if the diet had been finely ground, but when the diet had been less finely milled material (wheatfeed) they contained in addition many hard white granules which were easily identified as pieces of endosperm (semolina).* The original diet contained few or none such granules but did contain pieces of endosperm with adhering bran. It was concluded that the larvae had torn off the bran, eaten it, and rejected the white endosperm.

To proceed further it was necessary to have a more carefully defined diet. Through the courtesy of the Cereal Research Station of the Research Association of British Flour-Millers, samples of partially milled wheat were available. The fine material, which passed through a sieve of 40 meshes to the inch, was rejected, and the larger particles examined. They were of two kinds, clean white endosperm and 'mixed' particles of endosperm with adherent bran. With this material the following experiments were performed.

* Technical terms of the flour milling industry—wheatfeed, semolina, throughs, overtails, etc.—are used as defined by J. F. LOCKWOOD (1948).

(i) Larvae were offered the sieve overtails directly, i.e. a mixture of the two kinds of particles. At twice-weekly intervals the diet was examined, noting particularly the proportion of 'mixed' particles. This soon began to fall, and eventually no more particles of this kind could be found. This experiment does not distinguish between the following two possibilities: on the one hand that these particles were completely eaten and, on the other hand, that the bran was torn off and eaten and the endosperm rejected.

(ii) The two sorts of particles were separated by hand sorting under binoculars using glass needles. Larvae were offered only 'mixed' particles. Again the diet was examined at twice-weekly intervals, this time searching for clean white granules, which in these circumstances could only arise by the larvae tearing off the bran. Such granules were found, but never in great numbers at any stage from the start until nearly all the food was eaten. It was concluded that larvae could tear off the bran, but that the most favoured diet was one containing a fairly high proportion of endosperm.

(iii) Larvae were offered endosperm particles only. These were from another source and their average size was rather smaller, all passing through a sieve of 30 meshes to the inch, but overtailing an 80 mesh; 10 mg contained about 400 granules. This diet would be equivalent to white flour, were it not for the unknown additions which commercial white flour contains. But in any case granules were preferable, for then the amount of diet eaten could be determined accurately; in the process of removing frass from white flour by the tapping technique substantial and indeterminable losses of flour are caused by adherence to the paper. The larvae used in this experiment initially weighed between 20 and 30 mg, and each was separately transferred to a tube of granules immediately after moulting. Growth was normal at first and continued so for about two instars, but it then ceased. Regular weighings of the diet, after defrassing, showed that the larvae were no longer eating the granules. Care was taken always to weigh the diet immediately after removal from the constant temperature and humidity room, before it lost weight in equilibrating with the lower humidity of the laboratory. A typical set of figures for a single larva is the following: eaten during first week, 22 mg; during second, third, and fourth weeks combined, 30 mg; during fifth and sixth weeks combined, 4 mg. The main variation from one insect to another lay in the length of the initial period of normal growth which was particularly variable if white flour was substituted for granules.

The question arises how far this loss of appetite is due to nutritional deficiency. In mammalian nutrition there is frequently a vicious circle, malnutrition leading to a further loss of appetite. There is evidence that in insects this effect is often negligible. In this work *Tenebrio* larvae which had been completely starved for several weeks resumed feeding *immediately* on being offered food, and similarly the larvae which had lost appetite for endosperm ate bran immediately it was offered. This would hardly have been the case if the loss of appetite had been caused by malnutrition. The larva described in the last paragraph, at the conclusion of the 6 weeks on endosperm, received 100 mg coarse bran flakes crudely mixed

VOL. 4 1960

into the previous diet. This was completely eaten in 16 days, but in addition a further 40 mg endosperm was eaten, more of this alone than had been eaten in the previous 5 weeks. Very large quantities of frass were passed during this period, in contrast to the endosperm only period, when the total frass passed over the whole 6 weeks was only 7 mg. The low roughage content of endosperm was indeed considered as a possible cause of the loss of appetite, but experiments described in the next section seem to prove that it is not.

Conclusions from this set of experiments are as follows. The feeding response evoked by endosperm alone progressively diminishes with time; presence of bran maintains or restores the stimulus. When bran and endosperm are both present the preferred diet is a mixture of the two.

B. *Discrimination in mixtures of parts of grain with powdered cellulose*

In search of a substance which might fail entirely to evoke a feeding response, cellulose was the first substance to be examined. Balston's 'Genuine Whatman Cellulose Powder for Chromatography' was used. It was fine enough to pass through an 80 mesh sieve and its physical properties, including density, would not be likely to lead to its rejection. It also took up moisture from a humid atmosphere, though very much less so than flour. Whether this might contribute to non-acceptance is not clear; larvae of *Tenebrio* possess receptors for humidity (ROTH and WILLIS, 1951), whereas adults of *Tribolium castaneum*, placed between two samples of flour of differing moisture content, attempt to reach the more humid (WILLIS and ROTH, 1950), but it does not appear that the results described below can be invalidated for this reason.

Although it has been found in this laboratory (DADD, 1956) that starving adults of *Tenebrio* will eat moist cellulose, throughout the present work larvae which have been transferred at ecdysis or which have been deprived of food since their last ecdysis have never eaten this preparation when it has been offered unmixed with any other material. This statement is based on the fact that no frass is passed, neither is undigested cellulose found in the gut on dissection. When the diet is examined for frass by the tapping method, objects which appear to the naked eye to be pellets of frass are sometimes found, but when viewed under magnification they are clearly seen to be only aggregates of uningested cellulose held together by some sticky secretion presumably from the insect's mouth. An important qualification to the statement that cellulose is not eaten is that transfer must be made before the larva has commenced to eat again since its last ecdysis; larvae transferred during the middle of the eating period continue to eat for a short while.

Having established that powdered cellulose alone is not eaten, it was possible to mix acceptable foods into cellulose and observe the response. Larvae were left in cellulose for a preliminary period, 3–8 days usually, though longer periods have made no difference to the results, and after checking the absence of frass some other material was stirred into it. In the first series of experiments the material added was sieved wholemeal flour with addition of 5 per cent dried yeast, that is, normal diet except for the exclusion of middlings, so that the whole material

would pass through an 80 mesh sieve and make an intimate mixture with the cellulose. Mixtures of various flour contents from 50 per cent down to 2·5 per cent were used. In every case the larvae responded by eating, the weight of the larva rising in a single day while frass was passed on the second day. This frass, removed by tapping, was almost pure white and had a very low uric acid content, the lowest figure being 1 per cent as against about 12 per cent for the nutritive elements of this diet without the cellulose. (The uric acid content of frass naturally depends on the amount of roughage in the diet, ranging from 6 per cent on bran to 18 per cent on endosperm. In starvation, when the frass is almost wholly derived from the Malpighian tubules, it may rise to 50 per cent.) Clearly it consisted mainly of cellulose. The immediate nature of the response to the introduction of quite small quantities of flour into the cellulose was very marked.

The amounts of frass passed were astonishingly large. A small selection of actual figures is given in Table 1. The larvae in these experiments did not reach the stage of pre-moult, in which feeding and passage of frass are suspended, and frass production was probably spread evenly over the period. The frass consisted

VOL. 4 1960

TABLE 1—GAINS IN BODY WEIGHT AND QUANTITIES OF FRASS PASSED AFTER FEEDING LARVAE OF *Tenebrio molitor* FOR SHORT PERIODS ON MIXTURES OF WHOLEMEAL FLOUR AND CELLULOSE

Percentage of flour in mixture	Weight of larva (mg)			Frass passed (mg)	Period of observation (days)
	Initial	Final	Gain		
50	30	45	15	41·5	7
28·5	22	36·5	14·5	44	9
21·7	53	71	18	62	9
20·0	34	43·5	9·5	24·5	3
16·3	53	61	8	22·5	5
15·1	71	83	12	67	10
12·8	82	96·5	14·5	78·5	6
8·1	27	34·5	7·5	35	5

of discrete pellets, roughly round though more accurately barrel-shaped. Pellets from larvae weighing 60 mg weighed about 60 to the milligram, so that an excretion rate of 6 mg per day, which Table 1 shows was frequent, would be 360 pellets a day or a pellet every 4 min. It is difficult to believe that this rate could be very much further exceeded.

In fact when these conditions were maintained for longer than say 2 weeks the larvae died. The cause of death is a matter for speculation. Such a rapid passage of material through the gut would put a severe strain on the water-reabsorbing mechanism of the hindgut, but desiccation was by no means the only cause of death, because other larvae on the same diet but given access at frequent intervals to a pad of wet cotton wool (from which they drank large quantities up to 20 mg of water) also died. The answer may lie in the problem of producing the peritrophic

membrane. *Tenebrio* produces a peritrophic membrane of the type which is delaminated from the surface of the cells of the midgut epithelium. These membranes are produced intermittently with alternating periods of secretion and recuperation, and in zygopterous larvae WATERHOUSE (1954) has shown that the intervals between delaminations are shorter in fed than in starving insects. If this observation is of general validity (of which there is not yet proof) increasing speed of passage of food down the gut might stimulate an increased frequency of delaminating a membrane until the limit was reached where the mechanism broke down. There is a very well-marked difference in the texture of the frass of *Tenebrio* on different diets. As already described, on diets containing indigestible matter the frass takes the form of discrete pellets, but on flour without middlings no pellets are formed, the frass consisting of long twisty threads of which lengths of a centimetre may protrude in a coil from the insect's anus. The most likely explanation of this is also one relating the peritrophic membrane with the rate of passage of food through the gut; the smallness of the indigestible matter so slows the rate of passage that successive delaminations overlap and fuse into a continuous thread.

Leaving the subject of frass production and returning to that of eating, interest was next directed to the response of larvae offered mixtures in which the nutrient to cellulose ratio was very low. In order to obtain the same amount of nutrient the larvae would need to eat more, but in fact they ate less, as measured by frass production which did not exceed 3 mg per day on a mixture containing only 2·5 per cent flour/yeast. This was in full conformity with the idea that the response is caused by a feeding stimulus and is not related to the degree of 'hunger' in the sense of nutritional deficiency. It was also noted that on this very low nutrient diet the response, as measured by frass production, became negligible after about 7 days, could be re-evoked by a further small addition of flour, and that the new response also worked itself out in turn.

Conclusions from these results are as follows. (i) Cellulose is unable to evoke a feeding stimulus even in starving larvae. (ii) When flour is mixed in to cellulose a feeding response is evoked, weak when the flour content is very low, but rising with increased flour content until it appears to be limited only by the rate at which indigestible matter can be eliminated. (iii) When the mixture consists wholly of material fine enough to pass an 80 mesh sieve the larvae cannot effect complete discrimination, that is, they cannot avoid eating cellulose in addition to the nutritive constituent.

On the other hand the results for low flour contents are difficult to explain unless it is assumed that there has been some degree of discrimination; the most logical reason for a new response is that the substance responsible for the previous response has been removed. Fortunately this point can be resolved by the aid of a little calculation without need for further experiments. Now the amount of food required to be eaten in order to produce 1 mg gain in tissue weight is known from normal growth measurements; it is about 4 mg for diets containing middlings, but in the absence of this is nearer 3·3 mg. This gain in tissue weight is the total gain in weight of the larva minus a correction for the weight of cellulose remaining in

the gut; no accurate figure can be given for this, but it has been here assessed as $2\frac{1}{2}$ mg since this is the average amount of extra frass passed on starving the insect. Hence the expression [(total weight increase in mg $-2\frac{1}{2}) \times 3{\cdot}3$] is a measure, albeit somewhat rough, of the amount of flour eaten during the experiment. About one-eighth of this intake will turn up as frass, the rest of the frass will be the cellulose eaten; an expression for the latter is therefore [total frass $-(\frac{1}{8} \times$ flour intake)]. A ratio of flour eaten to cellulose eaten can thus be found and compared to the ratio in which the two components were present in the mixture offered. This comparison, for the same insects to which Table 1 relates, is expressed in the form of a histogram in Fig. 1. It shows that at least as far as mixtures with low flour

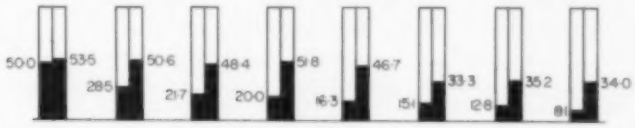


FIG. 1. Partial discrimination between wholemeal flour and cellulose effected by larvae of *Tenebrio molitor*. The left-hand column in each case represents the proportions of flour (black) and cellulose (white) in the mixture offered; the right-hand column shows the estimated proportions of each actually ingested.

content are concerned, there is very considerable discrimination, which reached its height with the larva offered 25 mg flour in 500 mg total material and which gained $8\frac{1}{2}$ mg weight. Using the above formula, $(8\frac{1}{2} - 2\frac{1}{2}) \times 3{\cdot}3$ is 20 mg, which means that the larva succeeded in picking out 80 per cent of the flour content at a cost of unavoidably eating about 8 per cent of the cellulose content.

To the previous conclusions must therefore be added: (iv) Larvae can effect partial discrimination in mixtures where the proportion of the taste-stimulating substance is low.

The next experiments were directed to discover whether larvae could effect complete discrimination if there was a distinct difference in particle size between the stimulating and non-stimulating constituents.

(i) Bran flakes were stirred into cellulose powder to make a mixture as dispersed as possible, and larvae were offered this mixture. Copious frass was produced, but it was exactly the same gingerbread shade of brown as frass passed on bran alone. Although not a conclusive result it appeared that discrimination was complete and that no cellulose had been eaten.

(ii) Granules of endosperm were similarly stirred into cellulose powder and the mixture offered to larvae. Only a very small amount of frass was passed, and this did not consist of pellets but of twisty threads nearly black in colour, exactly resembling that produced by larvae feeding on endosperm granules alone. This was the conclusive evidence, lacking in the previous experiment, that discrimination was complete and that the larvae had rejected the cellulose absolutely.

The larvae in the last experiment, when left for a prolonged period in the mixture, showed the same behaviour as those offered endosperm alone, the feeding response becoming feebler and ultimately ceasing. Since the diet actually

being eaten was exactly the same, this was natural, but the observation provided the opportunity of testing the point, previously described as undecided, whether diminished response could be due to lack of roughage in the diet. The experiment was introduced of offering an intimate mixture of white flour and cellulose. The larvae in this mixture passed large quantities of cellulose-containing white frass, being unable to effect complete discrimination between these intimately mixed constituents. Nevertheless they also showed the response of loss of appetite. The conjunction of these two observations was taken as final proof that diminished response was not due to lack of roughage.

(iii) As a tailpiece to this series of experiments bran was loosely stirred into an already intimate mixture of flour and powdered cellulose, and the whole offered to larvae. A large quantity of frass was produced which was found on examination under magnification to be a mixture, some pellets being brown and some white. Evidently both parts of the diet were acceptable but were not eaten simultaneously.

C. *Attempted localization of the response-promoting substance in bran*

The name bran is used for the three outermost layers of the grain, the pericarp, testa, and nucellus, but commercial bran ordinarily contains in addition to these three all or part of the aleurone layer and some starchy endosperm.

Bran is ultimately eaten away completely by *Tenebrio* larvae and replaced by frass, but this does not of course prove that all parts of the bran are equally attractive. Evidence on this point must be sought by interrupting the experiment before all the bran has been eaten. A number of experiments have been tried where the larvae were removed at the stage where 80–90 per cent of the bran has been eaten. Not all the experiments have been successful, but sometimes it has been found that the residue at this stage is markedly different from the starting material, the flakes being much thinner and paler. Microscopic examination confirms that they consist of fewer layers, and the high proportion of pieces with hairs on the outer surface identifies these as pieces of pericarp from the stigmatal end of the grain, where there is a tuft of hairs called the brush. When sufficient of these thin pale flakes had been isolated, the reaction of larvae to them was more closely studied. Some larvae ate them readily enough, thus explaining why they were not found in all experiments, but others gave only a feeble response. One larva was left on such a diet for 9 weeks; over this period it ate 70 mg but it gained no body weight, in fact suffered a slight loss, and did not moult. At the conclusion it was transferred to normal bran and ate sufficient to pass 90 mg frass in 3 weeks and gain 20 mg in weight.

It was concluded that the response-promoting substance is located in the inner layers of the bran, either in the testa, nucellus, or aleurone layer.

Heating bran flakes for $2\frac{1}{2}$ hr at 140°C did not destroy the stimulating factor.

D. *Acceptability and non-acceptability of other nutrient substances*

The results so far prove that some constituent or constituents in the wheat grain, and particularly in a certain part of it, acts as a release substance for the

initiation and continuation of normal feeding in larvae of *Tenebrio*. This substance may be something devoid of any nutritional value or it may be itself a nutrient. THORSTEINSON (1958) has made it clear that no sharp distinction exists between the former group (token stimuli) and substances which are both stimulants and nutrients, to which group he has given the name 'sapid nutrient'. Some preliminary experiments were therefore carried out with simple proteins, carbohydrates, and fats, to investigate any attractant effect they might have, and with some foods not of cereal origin which have been reported by other workers as being eaten by *Tenebrio*.

(i) *Proteins*. Commercial samples of casein and egg albumin were used, and also gluten (presumed to be a mixture of glutenin and gliadin). Where necessary the material was ground until the particles passed through an 80 mesh sieve. These proteins were first offered alone. Gains in body weight of only a few milligrams resulted, the small amount of frass passed had a high content (over 25 per cent) in uric acid, though this latter is also characteristic of starving animals. After a few days the insects began to lose weight. When the protein was offered in the form of a mixture with cellulose, there was never any large-scale ingestion of the latter. In some experiments starch was also included to offset any possible effect of overloading the digestive system with nitrogenous food, but it did not render the diet any more acceptable. Some of the insects in these experiments died rather soon, but others responded normally when later transferred to known acceptable diets.

(ii) *Carbohydrates*. Soluble sugars, particularly sucrose, are known to be very acceptable to many insects. BECK (1956) has shown that larvae of the European corn borer *Pyrausta nubilalis*, when offered the choice of several agar gels containing different concentrations of sucrose, always select the one with highest sugar concentration.

Sucrose–cellulose mixture containing 10 per cent of the former was offered to larvae preconditioned in the usual manner. The response was very variable as between individuals, in marked contrast to the experience with all the previously tested materials. Generally the diet was not initially acceptable, observations every 2 days revealing no frass for periods of as long as 2 weeks, when an abrupt reversal of behaviour occurred, resulting in eating on a scale to produce 5 mg frass per day. But sometimes this reversal occurred after only 4 or 5 days, and with a few larvae the mixture was acceptable from the start.

No other soluble sugar was investigated, but the polysaccharides starch and dextrin were found to give results very similar to the ones described for sucrose.

(iii) *Fats*. It is difficult to study the acceptability of fat because it is impossible to mix fat with powdered cellulose without greatly altering the physical texture. An ether solution of fat obtained by shaking fishmeal, which is an acceptable food, with ether, was poured on to cellulose powder and the ether allowed to evaporate. The material left was no longer a powder and although it could be forced through a fine sieve it reagglomerated as soon as it was through. Larvae which were placed in this material passed no frass. After a few days their weight began to decline,

VOL. 4 1960

and soon this decline grew to catastrophic proportions. The larvae lost more than half their total weight and eventually died. The most likely explanation of this would seem to be that the cuticular wax layer partially dissolved in the dietary fat so that the insects lost water and became desiccated; this has not however been verified. A small amount of this fatty cellulose was rubbed up with a large amount of flour–cellulose mixture containing 10 per cent flour; larvae which were feeding on the latter continued to feed on the new mixture, in spite of the fact that the physical texture of the new mixture was determined by the fatty element in it, causing it to clump; frass production continued at the same rate. The fat evidently did not act as a repellent.

The attractiveness of wholemeal wheat flour to *Tenebrio* larvae does not appear to be attributable to a general attractiveness of either proteins, carbohydrates, or fats.

(iv) *Some foods reported by other workers as acceptable.* LECLERCQ (1948) described attempts to rear *Tenebrio* larvae on fishmeal and on dried yeast. He chose these substances in order to study a diet of high protein and very low carbohydrate content; in the present connexion the important point is that they were accepted and eaten. This was confirmed and the following extra points established.

The fishmeal was fibrous in structure and could not be made into a fine powder. Although only particles passing a 50 mesh sieve were used, the mixture with cellulose was never very intimate and the constituents could be separated by tapping. It seemed probable that the larvae would be able to distinguish between the two constituents and reject the cellulose. But it proved otherwise. Larvae offered this diet passed a copious frass of a pale cream colour containing much cellulose. Dried yeast, being a very fine powder, made an extremely intimate mixture with powdered cellulose, and larvae offered such a mixture passed very large quantities of frass.

Leclercq's observation that these two substances are inadequate for rearing larvae to the stage of pupation was confirmed. This was not due to any diminution in intensity of the feeding response with time, as had been the case with endosperm. On the contrary, the high rate of feeding, as determined by frass production, continued for long periods. Larvae placed in these diets when weighing 20 mg, if allowed occasional access to a wet pad from which they drank water, ate voraciously for periods of 12–16 weeks, reaching a weight of 70 mg and moulting 4–6 times. Then their condition changed quite suddenly, they ceased eating, and died in a few days. Towards the end of their life their outward appearance became abnormal, in that they were peculiarly translucent. This was found to be due to an almost complete absence of fat deposits in the fat body. MACLEOD (1941), who showed that *Tenebrio* larvae often recovered completely from periods of total starvation to give rise to normal adults, concluded that recovery never occurred if the larvae had consumed their whole supply of reserve fat. Some metabolic abnormality related to fat synthesis thus appeared to be the cause of death in these larvae. It was not mere deficiency of carbohydrate, because in some experiments starch was added to the other two constituents; the result was a speeding up of all the processes,

greater frass production, faster increase in body weight, and higher maximum weight reached, but earlier death of the larvae.

In the terminology of THORSTEINSON (1958) therefore, fishmeal and dried yeast contain a powerful 'acceptant', but they also have a toxic effect, not necessarily due to the same substance.

No indication can be given as yet as to the chemical nature of the acceptant in either of these products. The position regarding the acceptant in wheat grain is in fact similar, the information being mainly of a nature to exclude certain of the nutrients. This work claims however to establish that *Tenebrio molitor* in its behaviour pattern of feeding is to be regarded as not essentially different from other phytophagous insects.

REFERENCES

BECK S. D. (1956) The European corn borer, *Pyrausta nubilalis* (Hubn.) and its principal host plant—II. The influence of nutritional factors on larval establishment and development on the corn plant. *Ann. ent. Soc. Amer.* **49**, 582–588.

DADD R. H. (1956) Proteolytic activity of the midgut in relation to feeding in the beetles *Tenebrio molitor* L. and *Dytiscus marginalis* L. *J. exp. Biol.* **33**, 311–324.

DETHIER V. G. (1953) Host plant perception in phytophagous insects. *Trans. 9th Int. Congr. Ent., Amsterdam* **2**, 81–88.

FRAENKEL G. (1958) The chemistry of host specificity of phytophagous insects. *4th Int. Congr. Biochem. Vienna*, Symposium No. XII. In press.

FRAENKEL G. and BLEWETT M. (1943) The natural foods and the food requirements of several species of stored products insects. *Trans. R. ent. Soc. Lond.* **93**, 457–490.

FRAENKEL G., BLEWETT M., and COLES M. (1950) The nutrition of the mealworm, *Tenebrio molitor* L. *Physiol. Zool.* **23**, 92–108.

LECLERCQ J. (1948) L'importance des glucides dans la nutrition des larves de *Tenebrio molitor* L. *Arch. int. Physiol.* **56**, 28–34.

LOCKWOOD J. F. (1948) *Flour Milling*. 2nd ed. Northern Publishing Co., Liverpool.

MACLEOD G. F. (1941) X-ray studies of starving mealworm larvae. *Ann. ent. Soc. Amer.* **34**, 696–701.

ROTH L. M. and WILLIS E. R. (1951) Hygroreceptors in Coleoptera. *J. exp. Zool.* **117**, 451–487.

THORSTEINSON A. J. (1953) The chemotactic responses that determine host specificity in an oligophagous insect (*Plutella maculipennis* Curt.). *Canad. J. Zool.* **31**, 52–72.

THORSTEINSON A. J. (1958) The chemotactic influence of plant constituents on feeding by phytophagous insects. *Ent. exp. appl.* **1**, 23–27.

VALENTINE J. M. (1931) The olfactory sense of the adult mealworm beetle, *Tenebrio molitor* L. *J. exp. Zool.* **58**, 165–228.

WATERHOUSE D. F. (1954) The rate of production of the peritrophic membrane in some insects. *Aust. J. biol. Sci.* **7**, 59–72.

WILDE J. DE (1958) Host plant selection in the Colorado beetle larva. *Ent. exp. appl.* **1**, 14–22.

WILLIS E. R. and ROTH L. M. (1950) The attraction of *Tribolium castaneum* to flour. *J. econ. Ent.* **43**, 927–932.

VOL. 4 1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 92 *to* 101. *Pergamon Press Ltd., London. Printed in Great Britain*

THE EFFECTS OF FREEZING TEMPERATURES ON THE DEVELOPING EGG OF *LEPTOHYLEMYIA COARCTATA* FALL. (DIPTERA, MUSCIDAE) WITH SPECIAL REFERENCE TO DIAPAUSE DEVELOPMENT

M. J. WAY

Rothamsted Experimental Station, Harpenden, England

(*Received* 24 *June* 1959)

Abstract—The later stages of diapause in the egg of *L. coarctata* are completed rapidly at −20°C to −24°C. At −24°C the rate of diapause completion is about 180 times faster than at −6°C and about 80 times faster than at 3°C. These results suggest that there are at least two phases in diapause development—an initial synthesis, perhaps of neurohormone, which will not occur at −18°C to −24°C. This is followed by a process ending in release of the synthesized material which is greatly accelerated by relatively short exposures at −20°C to −24°C; longer exposures kill the eggs, probably because they freeze.

THE eggs of *Leptohylemyia coarctata* Fall. are laid in August and hatch in the following January to March. In natural conditions they may experience temperatures ranging from over 30°C in August to below −5°C in January and February. There are three stages of development (Way, 1959). First, prediapause morphogenesis which can occur within constant temperature limits of just below 3°C to over 30°C; this merges into diapause development (ANDREWARTHA, 1952) with an upper constant temperature limit of about 12°C and an optimum of about 3°C which is followed by postdiapause development with limits of 0°C and over 30°C. Preliminary experiments on the lower limit for diapause development showed that it becomes very slow at −6°C compared with at 3°C but may be accelerated by a temperature of −20°C (WAY, 1959). This paper describes further work on the effects of low temperatures, and discusses the results in relation to current theories on the mechanism of diapause development.

METHODS

Eggs of *L. coarctata* were obtained as described elsewhere (WAY, 1959); 40–100, in batches of 10 or 20, sandwiched between two small pieces of parachute nylon, were used for each treatment and were sometimes kept in soil outdoors until needed for an experiment. During experiments each batch was kept on a pad of damp cotton wool in corked 2 inch × 1 inch specimen tubes or in 4 inch long × ¼ inch wide sealed glass tubes. The latter were used in experiments where immersion in liquids was necessary, but the eggs were transferred to corked specimen tubes immediately after the immersion treatment was complete. For treatment in constant temperature cabinets, the tubes of eggs were usually wrapped in cotton wool and kept inside lead-lined aluminium containers.

The effect of a treatment on diapause development or on survival was usually determined by transferring 40–100 eggs either to 20°C or to 5°C. Eggs in which diapause was complete hatched within 12 days at 20°C (WAY, 1959). In experiments to see whether a treatment which did not end diapause had accelerated or delayed diapause development, 5°C was used as the incubation temperature, and the hatched eggs were counted weekly.

Insulated cabinets were used for constant temperatures of 3, −2, −6, −10, and −20°C. In each, the air was drawn across the main chamber after being cooled or heated in a separate conditioning chamber. The temperature was regulated by a Sunvic bimetallic thermostat type T.S.7 operating two type F102–3 Sunvic hot wire relays which operated the heaters and coolers alternately with no differential. Cooling was by one or two 95A Electrolux refrigerator units and heating by five tungsten lamps, the voltage of which was adjusted to give frequent temperature oscillations with the smallest possible amplitude. As the efficiency of the refrigerator units depends on the ambient temperature, the cabinet at −20°C was kept in a constant temperature room at 0·5°C to ensure that the required temperature was maintained during summer. Continuously recording mercury-in-steel thermographs showed temperature fluctuations of under ± 0·3°C in the circulating air of cabinets set at temperatures varying from 3 to −20°C. However, there was no fluctuation when the recording bulb was fixed inside the lead-lined containers used to store the eggs. The temperature also remained constant at −18°C in a similar can kept on the bottom shelf of a Deep Freeze (J. and F. Hall—model B8536LK).

VOL. 4 1960

A temperature of −22°C (−21·7 ± 0·6°C) was obtained with a eutectic freezing mixture of 23 per cent anhydrous sodium chloride in water. The eutectic temperature was maintained by adding a few lumps of solid carbon dioxide twice daily to about 4 l. of the mixture in a thermos vessel. Boiling methyl chloride was used to get a temperature of −24°C (about −23·8 ± 0·2°C). About a litre was put in a three-necked 2 l. flask to one neck of which was attached a spiral copper condenser cooled by solid carbon dioxide in ethyl alcohol. This returned the methyl chloride to the flask which was sufficiently insulated to keep the methyl chloride simmering gently. Sealed tubes containing eggs were immersed in the methyl chloride and were attached by wires to a ground glass stopper in the centre neck of the flask. A thermometer was fixed in the third neck; temperature was not recorded continuously.

The undercooling point of diapausing eggs of *L. coarctata* was determined using two 40 gauge copper–constantin thermocouples, one in ice and water at 0°C and the other, for recording the egg temperature, inserted through a glass tube into a corked 2 inch × 1 inch specimen tube which was fixed inside a corked 250 ml conical flask. This was cooled by placing it inside an empty 1 l. beaker immersed almost to its rim in ethyl alcohol containing solid carbon dioxide at a temperature of −72°C. This system of insulation ensured comparatively slow cooling at a constant rate, the temperature of the thermocouple falling to −20°C 8–9 min after the flask was transferred from room temperature to the beaker. An egg, which is about 1·4 mm long and 0·4 mm wide, was stuck to the thermocouple either by 'Vaseline' or by water. Contact water did not alter the undercooling point of the embryo providing the vitelline membrane was intact (SALT, 1936). The falling temperature was observed on a previously calibrated Cambridge galvanometer with adjustable back-off potentiometers, and the freezing of the egg was marked by the point where there was a sharp rise of 4–6°C caused by release of latent heat of freezing. I am indebted to Dr. R. W. SALT for the main details of this method.

RATE OF DIAPAUSE TERMINATION AT TEMPERATURES BELOW 0°C

Eggs laid on 12–15 August were kept in soil outdoors. On 11 November when they had been in diapause for about 8 weeks, batches were transferred to 3, −6, −18, −22, and −24°C. At appropriate intervals 40–80 eggs were removed from

each temperature and incubated at 20°C for 12 days and the egg hatch recorded. Fig. 1 shows that diapause was completed very quickly at −24°C, 50 per cent of the eggs hatching after only 6 hr and 88 per cent after 24 hr at this

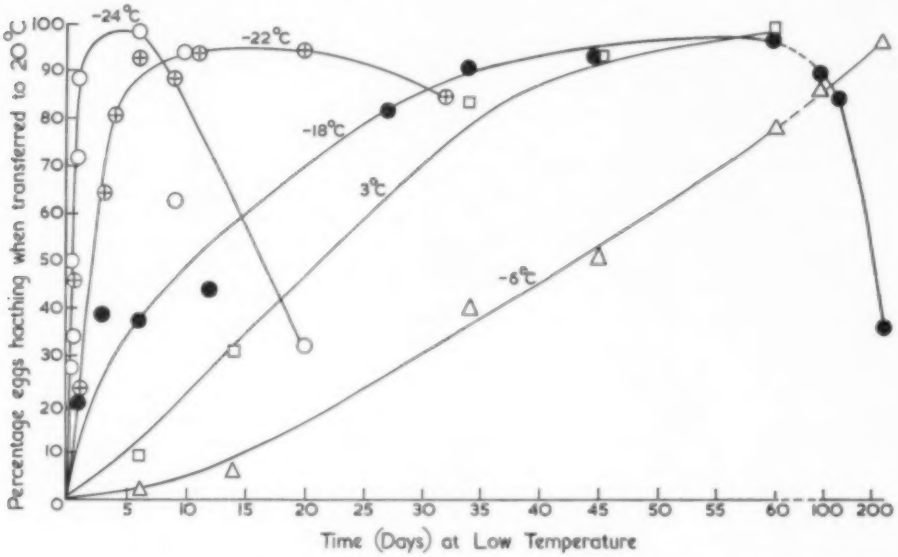


FIG. 1. Effect of varying exposures to low temperatures on the completion of diapause in *L. coarctata*.

temperature. About 20 days and 45 days were needed to end diapause in 50 per cent of eggs kept at 3°C and −6°C respectively. Thus at −24°C diapause was completed about 80 times faster than at 3°C and about 180 times faster than at −6°C; also, it was about 40 times faster at −24°C than at −18°C.

Fig. 1 shows that eggs were harmed when kept at a low temperature beyond the time needed to complete diapause. Thus 98 per cent hatched after 6 days at −24°C but only 32 per cent after 20 days. Similarly, 97 per cent and 36 per cent hatched after 63 and 206 days respectively at −18°C. Some of the embryos which failed to hatch were alive after 20 days at −24°C. They were flaccid and inactive and soon died.

In another experiment eggs laid on 14–23 August were kept in soil outdoors until 10 October. Batches were then transferred to 5, −6, −10, and −20°C. Sixty eggs were taken from each temperature after 7 days at −20°C and after 28 days at −6, −10, and −20°C. They were then incubated at 5°C, not at 20°C as in the previous experiment, and were examined weekly. There were more non-viable eggs than in other experiments, the percentages which failed to hatch in each treatment being: 5°C throughout = 24; −6°C = 25; −11°C = 30; −20°C for 7 days = 21; −21°C for 28 days = 36. The rates of egg hatch as

cumulative percentages of the totals which hatched in each treatment are shown in Fig. 2. Diapause development was accelerated by keeping the eggs for 7 and 28 days at −20°C compared with continuous treatment at 5°C, but was slowed

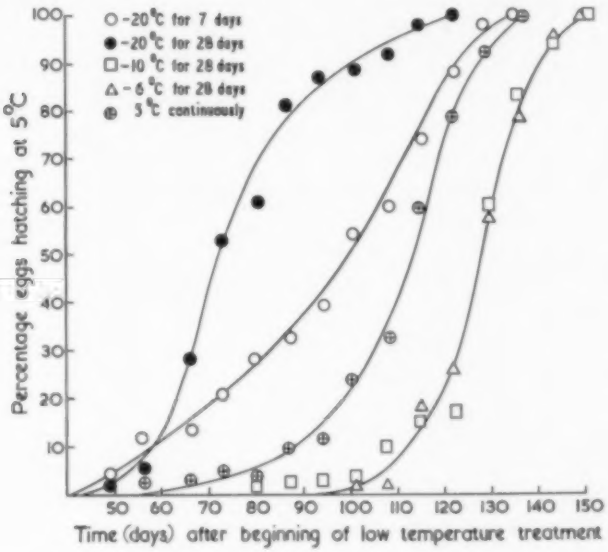


FIG. 2. Rates of hatch of diapausing eggs of *L. coarctata* kept at 5°C after treatment for 7 and 28 days at various low temperatures.

by 4 weeks at −6°C and −10°C. Thus the accelerating effect of storage at −20°C does not depend on the eggs being transferred subsequently to a relatively high incubation temperature.

TIME OF RESPONSE TO VERY LOW TEMPERATURE

Freshly laid eggs were kept at 20°C and at 5°C for 20, 50, 81, and 114 days, and were then removed to −18°C for 28 days followed by incubation at 20°C for 12 days. Table 1 shows that eggs must be kept for 50–80 days at 5°C before the

TABLE 1—THE EFFECT OF 28 DAYS EXPOSURE TO −18°C AT DIFFERENT STAGES IN DIAPAUSE DEVELOPMENT OF *L. coarctata* AT 5°C AND 20°C AS SHOWN BY THE PERCENTAGES WHICH HATCHED WHEN SUBSEQUENTLY INCUBATED AT 20°C

Initial temperature	Days at initial temperature before treatment at −18°C			
	20	50	81	114
20°C	6	7	26	—
5°C	0	28	72	94

treatment at −18°C became effective in ending diapause. This confirmed preliminary experiments in which exposure to −20°C for 1–4 weeks at the beginning of diapause development did not end diapause. It is significant that exposure to −18°C also ended diapause in some of the eggs kept previously at 20°C.

These results suggest that there are at least two phases in diapause development; the first proceeds relatively quickly at about 5°C and is also completed in some eggs at 20°C; it will not proceed at −18°C or −20°C. The second phase proceeds most quickly at −18°C to −24°C and, as shown previously (WAY, 1959), is also completed at 5°C but not at 20°C.

SIGNIFICANCE OF UNDERCOOLING AND FREEZING

Insects, especially when in diapause, frequently undercool to between −20°C to −30°C before they freeze (SALT, 1936). Although very few species can survive after being frozen, it seemed possible that freezing might be the cause of the rapid completion of diapause in the *L. coarctata* egg exposed to from −18 to −24°C. Experiments were therefore done to determine the undercooling point. The method, already described, showed that there was remarkably little variation in the undercooling point of different eggs. For example, in one experiment, 20 eggs had undercooling points between −25·3 and −28·3°C with 16 between −26·2 and −27·7°C. The mean in three different experiments done in 1956 was −27·1°C. In an experiment one year later the mean was −28·1°C, the variation being from −27·2 to −30·0°C.

These results show that eggs do not freeze immediately when cooled to −24°C. SALT (1950) has shown, however, that insects held in an undercooled state eventually freeze, so that eggs of *L. coarctata* might be expected to freeze after a few days at −24°C, after a few weeks at −22°C, and after a few months at −18°C. This seems a likely explanation of the lethal effect of prolonged exposure to these temperatures (Fig. 1) rather than of their action in ending diapause. A further experiment confirmed that freezing is lethal; 19 eggs were cooled to −26·5°C and were then allowed to return to a temperature of 20°C. A temperature record kept throughout cooling and re-warming showed that 8 eggs froze. Seven days later all the eggs were dissected and the embryos examined; those which froze were dead, and the others were normal. Therefore, diapause completion at from −18 to −24°C occurs without the eggs freezing.

MORTALITY DURING STORAGE AT LOW TEMPERATURE

Fig. 1 shows that eggs of *L. coarctata* begin to die, probably because they freeze, when kept for more than about 6 days at −24°C or for more than 60 days at −18°C. This was investigated in a further experiment in which possible complications from processes of diapause completion were avoided by using eggs kept at 5°C and latterly at −2°C until 24 January when diapause was almost complete. By this time only 9 eggs, including 7 which were dead, failed to hatch in a batch of 110 eggs incubated for 12 days at 20°C. Some eggs were kept at

$-2°C$ and others transferred on 24 January to -6, -18, and $-20°C$. Batches of 40–60 were removed from each temperature at appropriate intervals, and incubated at 20°C. Twelve days later they were carefully examined. The percentages (p^1) which had died in each treatment were corrected for the 6 per cent natural mortality using the formula $p = (p^1 - 6)/(100 - 6) \times 100$ (FINNEY, 1952) where p = the corrected percentage mortality.

The time–mortality curves for eggs kept at the four temperatures are shown in Fig. 3. The times for 50 per cent kill were: about 50 days at $-20°C$ and 150 days at $-18°C$, whereas there was still no kill after 291 days at $-6°C$. These results conform with the suggestion that at $-18°C$ and $-20°C$ the eggs die because they freeze, whereas they would probably remain supercooled indefinitely at $-6°C$. Fig. 3 shows, however, that about half of the eggs died when stored for about

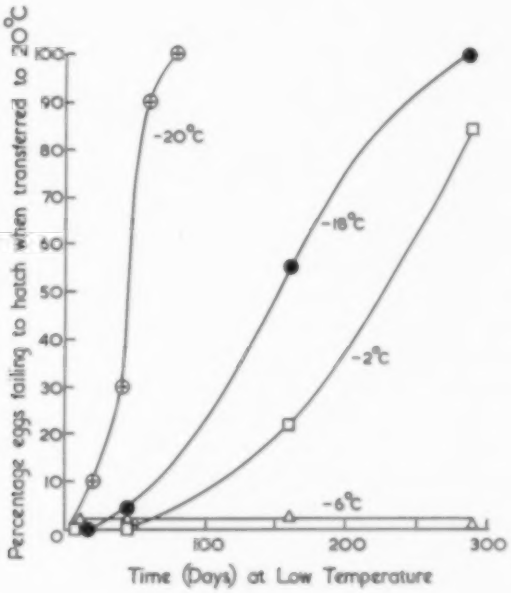


FIG. 3. Effect of storage at low temperatures on viability of post-diapause eggs of *L. coarctata*.

150 days at $-2°C$. The mortality cannot be caused by freezing but could be caused by toxic metabolic products which would normally be eliminated but which accumulate in an insect when it is kept below the temperature threshold for normal development, but above that which inhibits metabolism (WIGGLESWORTH, 1953). Such mortality is known to occur after completion of diapause in eggs of *Lymantria dispar* (KOZHANTSHIKOV, 1950) and in pupae of *Hyalophora cecropia* (WILLIAMS, 1956).

VOL. 4 1960

DISCUSSION

WIGGLESWORTH'S (1934) suggestion that diapause in some insects occurs when the brain or other endocrine centre fails to secrete a hormone which is necessary for further development is now well substantiated (LEES, 1955, 1956). The fully developed embryo of *Leptohylemyia coarctata* possesses a brain and endocrine system, and hormone failure may therefore induce its diapause. A different mechanism presumably exists in insects that diapause as undeveloped embryos (LEES, 1955); also this discussion relates to insects which diapause at the end of an instar—i.e. as fully developed embryos, as larvae about to undergo ecdysis, or as pupae just before metamorphosis. It may not be relevant to adult diapause which is associated with reproductive dormancy and not with arrested morphogenesis.

Comparatively little is known about the reactions during diapause development which culminate in the secretion of the brain hormone. LEES (1955) has pointed out that diapause development in most insects is favoured by moderately low temperature and is inhibited by high temperatures, and probably involves antagonistic synthetic and breakdown reactions in the brain with different positive temperature coefficients. VAN DER KLOOT (1955), WILLIAMS (1956), and SCHNEIDERMAN and HORWITZ (1958) suggest that diapause development in *Hyalophora cecropia* and *Mormoniella vitripennis* may comprise the steady accumulation of a particular substance presumably synthesized in the brain and is complete when the substance reaches a particular concentration. This may be a cholinergic substance as suggested by VAN DER KLOOT (1955) or it may be the product of the neurosecretory cells which has been shown to accumulate in the cells during diapause development or is transported to, and stored in, the corpora cardiaca until diapause is complete (SCHNEIDER, 1950; CHURCH, 1955; FRASER, 1957; HIGHNAM, 1958). However, the suggestion that diapause development is merely the steady accumulation of the substance to threshold concentration is not entirely compatible with evidence that the temperature optima for diapause development may vary greatly as diapause proceeds. Thus in *Mormoniella* (SCHNEIDERMAN and HORWITZ, 1958) aerobic warming (25–30°C) is initially favourable but subsequently it first reverses and then delays diapause development. Further, part way through diapause at 5°C, anaerobic warming at 25–30°C greatly accelerates diapause development whereas previously this treatment had no effect. Experiments described in this paper and elsewhere (WAY, 1959) show that in *Leptohylemyia coarctata* a temperature of 20°C is initially favourable for diapause development but from −18 to −20°C is unfavourable. Subsequently temperatures of from −18 to −24°C become favourable, whereas 20°C stops or reverses the process of diapause development. It also seems unlikely that diapause development in *Sitodiplosis mosellana* which may last for more than 12 years (BARNES, 1943) is merely a process of accumulation. Diapause development in the brain of the fully developed embryo of *Leptohylemyia coarctata* (Diptera, Muscidae) or in the larval *Sitodiplosis mosellana* (Diptera, Cecidomyidae) may well differ from that of a diapausing pupa of *Hyalophora cecropia* (Lepidoptera, Saturniidae), but it is significant that the pupa of *Saturnia pavonia* can complete

diapause development at temperatures of from – 15 to about – 18°C (DANILYEVSKY, 1949) which as in *L. coarctata* would almost certainly inhibit synthetic activity in the brain.

Neurosecretion normally comprises three processes (BURGIN, 1959): the synthesis of the hormone and its enclosure in a microvesicle, its transport within the neurosecretory elements, and finally its discharge. Perhaps the neurohormone is synthesized and accumulates during the early part of diapause development. In many insects this initial phase is completed most quickly at moderately low temperatures with an optimum usually in the range 5–15°C, and it is significant that such temperatures are experienced during September to November (i.e. during the early part of diapause) in all parts of the world where diapause occurs during a cool or cold winter (Fig. 4). In contrast, there may be big temperature

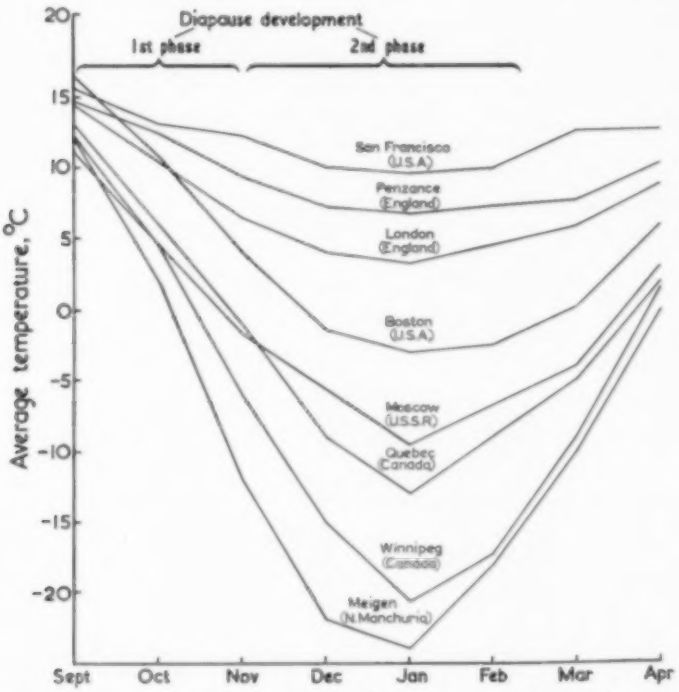


FIG. 4. Average monthly temperatures in different parts of the Northern Hemisphere showing that insects experience similar temperatures during the early part of diapause but not subsequently. Data from isotherms in BARTHOLOMEW and HERBERTSON (1899) except N. Manchuria (DANILYEVSKY, 1959).

differences during winter and early spring depending on geographical location, and, in insects the thermal requirements for the later stages of diapause development may be closely adapted to the prevailing temperatures (DANILYEVSKY, 1949; LEES, 1955; WAY, 1959). *L. coarctata* normally experiences a temperature of

VOL. 4 1960

about 0°C to 7°C during winter and therefore does not benefit from being able to complete the later stages of diapause at about the same rate at −18°C as at 3°C. However, this is evidence that in insects there are different mechanisms which provide the means for temperature adaptation. The neurohormone may perhaps continue to accumulate throughout diapause development in insects adapted to a comparatively mild winter, and diapause may end when it reaches a threshold concentration (SCHNEIDERMAN, 1956). However, as the later stages of diapause in *L. coarctata* can be completed at low temperatures which would inhibit synthesis, it seems that sufficient neurohormone to end diapause has already accumulated but its release is normally inhibited perhaps by a diapause hormone (HINTON, 1954) or more simply by an intracellular barrier. In species like *L. coarctata*, *Saturnia pavonia*, and *Gilpinia polytoma* (PREBBLE, 1941) this barrier is apparently broken down by adequate exposure to temperatures of from −10 to −24°C. Shock treatments such as brief exposures to 30–35°C in *Leptohylemyia coarctata* (WAY, 1959) or brief anaerobic warming at 25–30°C in *Mormoniella vitripennis* (SCHNEIDERMAN and HORWITZ, 1958) may similarly release the neurohormone by a physical as distinct from a chemical action. Further, the variations in duration and intensity of diapause in different insect species (see LEES, 1955) may depend on specific differences in the stability of the barrier withholding the neurohormone rather than on specific differences in requirements for synthesis of neurohormone or other substance in the brain.

Acknowledgements—My sincere thanks to Dr. A. U. SMITH and Dr. R. W. SALT for advice, to Dr. H. E. HINTON and Dr. A. D. LEES for criticizing the manuscript of this paper, and to Mr. R. GOODCHILD for valuable assistance.

REFERENCES

ANDREWARTHA H. G. (1952) Diapause in relation to the ecology of insects. *Biol. Rev.* **27**, 50–107.

BARNES H. F. (1943) Studies of fluctuations in insect populations—X. Prolonged larval life and delayed subsequent emergence of the adult gall midge. *J. Anim. Ecol.* **12**, 137–138.

BARTHOLOMEW J. G. and HERBERTSON A. J. (1899) *Physical Atlas*—III. *Atlas of Meteorology*. Bartholomew, London.

BURGIN A. S. V. (1959) Introduction. Symposium on neurosecretion. *Canad. J. Biochem. Physiol.* **37**, 307–308.

CHURCH N. S. (1955) Hormones and the termination and reinduction of diapause in *Cephus cinctus* Nort. (Hymenoptera: Cephidae). *Canad. J. Zool.* **33**, 339–369.

DANILYEVSKY A. S. (1949) The dependence of the geographical distribution of insects on the ecological peculiarities of their life cycles. (In Russian). *Ent. Oboz.* **30**, 194–207.

FINNEY D. J. (1952) *Probit Analysis; a Statistical Treatment of the Sigmoid Response Curve*. 2nd ed. Cambridge University Press.

FRASER A. (1957) Neurosecretory cells in the brain of the larva of *Lucilia caesar* L. *Nature, Lond.* **179**, 257–258.

HIGHNAM K. C. (1958) Activity of the brain/corpora cardiaca system during pupal diapause "break" in *Mimas tiliae* (Lepidoptera). *Quart. J. micr. Sci.* **99**, 73–88.

HINTON H. E. (1954) The initiation, maintenance, and rupture of diapause; a new theory. *Entomologist* **86**, 279–291 (1953).

KOZHANTSHIKOV I. W. (1950) Features of the hibernation and diapause of the gipsy moth (*Ocneria dispar* L.). (In Russian). *Dokl. Akad. Nauk*, SSSR **73**, 605–607.

LEES A. D. (1955) *The Physiology of Diapause in Arthropods*. Cambridge University Press.

LEES A. D. (1956) The physiology and biochemistry of diapause. *Annu. rev. Ent.* **1**, 1–16.

PREBBLE M. L. (1941) The diapause and related phenomena in *Gilpinia polytoma* (Hartig) —II. Factors influencing the breaking of diapause. *Canad. J. Res.* (D) **19**, 323–346.

SALT R. W. (1936) Studies on the freezing process in insects. *Univ. Minn. Agric. Exp. Stat. Tech. Bull.* **116**.

SALT R. W. (1950) Time as a factor in the freezing of undercooled insects. *Canad. J. Res.* (D) **28**, 285–291.

SCHNEIDER F. (1950) Die Entwicklung des Syrphidenparasiten *Diplazon fissorius* Grav. (Hym., Ichneum.) in uni-, oligo- und polyvoltinen Wirten und sein Verhalten bei parasitärer Aktivierung der Diapauselarven durch *Diplazon pectoratorius* Grav. *Mitt. schweiz. ent. Ges.* **23**, 155–194.

SCHNEIDERMAN H. A. (1956) Onset and termination of insect diapause. *Physiological Triggers*, 46–59.

SCHNEIDERMAN H. A. and HORWITZ J. (1958) The induction and termination of facultative diapause in the chalcid wasps *Mormoniella vitripennis* (Walker) and *Tritneptis klugii* (Ratzeburg). *J. exp. Biol.* **35**, 520–551.

VAN DER KLOOT W. G. (1955) The control of neurosecretion and diapause by physiological changes in the brain of the cecropia silkworm. *Biol. Bull., Woods Hole* **109**, 276–294.

WAY M. J. (1959) The effect of temperature, particularly during diapause on the development of the egg of *Leptohylemyia coarctata* Fall. (Diptera, Muscidae). *Trans. R. Ent. Soc.* **111**, 351–364.

WIGGLESWORTH V. B. (1934) The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera) —II. Factors controlling moulting and "metamorphosis". *Quart. J. micr. Sci.* **77**, 191–222.

WIGGLESWORTH V. B. (1953) *The Principles of Insect Physiology*. 5th ed. Methuen, London.

WILLIAMS C. M. (1956) Physiology of insect diapause—X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the cecropia silkworm. *Biol. Bull., Woods Hole* **110**, 201–218.

VOL. 4 1960

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

A. D. LEES: The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—II

S. E. LEWIS, J. B. WALLER, and K.S. FOWLER: The effect of DDT and of physically induced prostration on acetylcholine levels in the cockroach

G. BECHT, G. HOYLE, and P. N. R. USHERWOOD: Neuromuscular transmission in the coxal muscles of the cockroach

N. G. PATEL and A. G. RICHARDS: Proteolytic enzymes from the midgut of the housefly

V. B. WIGGLESWORTH and J. W. L. BEAMENT: The respiratory structures in the eggs of higher Diptera

H. AUTRUM and C. HOFFMANN: Diphasic and monophasic responses in the compound eye of *Calliphora*

L. HUOT and W. VERLY: Dosage du ^{32}P total d'une larve vivante de *Tenebrio molitor* L. (Coléoptère, Tenebrionidae)

H. E. HINTON: Plastron respiration in the eggs of blowflies

M. PASCAL and L. BAUD: Variabilité de la teneur en phosphore des ovocytes chez *Bombyx mori* L.

J. SIMPSON: The functions of the salivary glands of *Apis mellifera*

D. BURKHARDT: Action potentials in the antennae of the blowfly, *Calliphora erythrocephala*, during mechanical stimulation

H. D. PARK and J. BUCK: The relation of oxygen consumption to ambient oxygen concentration during metamorphosis of the blowfly, *Phormia regina*

S. D. BECK and W. HANEC: Diapause in the European corn borer, *Pyrausta nubilalis* (Hübn.)

P. W. MILES: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera, Lygaeidae)—II. Physical and chemical properties

G. F. HOWDEN and B. A. KILBY: Biochemical studies on insect haemolymph—II. Variations in reducing power with age and the effect of diet

M. B. SHYAMALA, K. SHARADA, M. G. BHAT, and J. V. BHAT: Effect of chloromycetin on the digestion and utilization of the constituents of mulberry by the silkworm

H. LANGER: Die Wirkung von Licht auf den chemischen Grundaufbau des Auges von *Calliphora erythrocephala*

B. J. SELMAN: On the tissue isolated in some of the larval appendages of *Sialis lutaria* L. at the larval–pupal moult

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 103 *to* 106. *Pergamon Press Ltd., London. Printed in Great Britain*

VARIABILITÉ DE LA TENEUR EN PHOSPHORE DES OVOCYTES CHEZ *BOMBYX MORI* L.

M. PASCAL et L. BAUD

I.N.R.A. Station de Recherches Séricicoles d'Alès

(*Received* 29 *April* 1959)

Abstract—The weight, total phosphorus content, and phosphorus content as a percentage of fresh weight was determined for oocytes in different positions in the ovarioles in the same female of *Bombyx mori* L. The weight and total phosphorus content increase up to the fifteenth oocyte, whereas phosphorus expressed as a percentage of fresh weight remains nearly constant although it may vary by about 15 per cent in neighbouring oocytes.

VOL.
4
1960

INTRODUCTION

L'ABSENCE d'alimentation des imago, de même que la simultanéité dans l'ovogénèse et la ponte chez *Bombyx mori* L., paraîtraient favoriser au maximum l'homogénéité entre les oeufs d'une même femelle. Cependant, de nombreux auteurs dont SECRETAIN et SCHENK (1942), ont signalé l'existence d'une variabilité notable du poids des oeufs d'une même ponte, considérés au moment de leur diapause embryonnaire; cette variabilité est essentiellement introduite par la différence de poids entre les premiers et les derniers oeufs pondus.

On a depuis précisé LEGAY (1958), que la même variabilité existait déjà au sein des tubes ovariques: les ovocytes les plus proches de l'oviducte commun étant les plus lourds, ce qui laisse supposer la possibilité d'un gradient nutritionnel.

Le présent travail a eu pour objectif de rechercher si certains constituants élémentaires de l'oeuf étaient soumis à une variabilité comparable à celle portant sur le poids.

Comme le phosphore intervient dans de nombreuses substances ayant un intérêt génétique et métabolique, nous l'avons choisi comme objet d'une première étude.

METHODE

Cinq cents ovocytes sont prélevés par dissection de deux femelles typiques (six ovarioles sur huit seulement ayant été retenus). Après séchage des chorions, chaque ovocyte est pesé au millième de milligrammes, numéroté selon la position qu'il occupait dans le tube ovarique et conservé.

On fait appel à la méthode de HORECKER *et al.*, citée par DELSAL et MANHOURI (1955), pour le dosage du phosphore total. Les quantités de réactifs utilisées pour un ovocyte sont:

0,5 ml de SO_4H_2 concentré,
0,5 ml de solution de molybdate d'ammonium à 5 pour cent,
0,5 ml du mélange réducteur en solution à
—0,2 pour cent acide amino-naphtol-sulfonique
—12 pour cent métabisulfite de sodium
—1,2 pour cent sulfite anhydre de sodium.

La minéralisation de chaque ovocyte dans les microkjeldahls de 10 ml est effectuée avec la quantité de SO_4H_2 mentionnée ci-dessus, en présence d'acide perchlorique selon DELSAL et MANHOURI (1955).

Les lectures sont faites au spectrophotomètre JEAN et CONSTANT à la longueur d'onde de 830 mμ, correspondant au maximum d'absorption.

RESULTATS

Les résultats des dosages concernant les différents tubes ovariques *considérés séparément*, sont comparables entre eux. Le graphique caractéristique ci-joint pris comme exemple, correspond aux ovocytes de l'un des tubes et comporte les trois courbes suivantes:

(a) poids de l'ovocyte en 1/1000 de milligrammes,
(b) phosphore total en γ,
(c) phosphore total exprimé en pour cent du poids frais, pour chacun des ovocytes en fonction de leur rang initial.

La courbe (a) montre jusqu'au quinzième ovocyte, une grande variabilité pondérale déjà signalée par LEGAY et liée, au stade ovocyte, à une chute rapide du poids. De plus, au delà du quinzième ovocyte, nous constatons une atténuation de la variabilité, en même temps que la pente générale de la courbe devient plus faible.

La courbe (b) permet de noter que le phosphore total décroît assez rapidement jusqu'au quinzième ovocyte, pour garder ensuite des valeurs alternant entre 1,7 γ et 2 γ. Or, l'erreur de dosage contrôlée atteignant seulement 0,04 γ, les différences quantitatives entre ovocytes successifs sont très souvent significatives. On doit cependant remarquer que chaque ovocyte ne peut faire l'objet que d'une seule analyse.

La courbe (c) est une droite sensiblement horizontale à partir du quinzième ovocyte. Le phosphore exprimé en pour cent du poids frais est compris entre 0,24 et 0,28 pour cent, c'est-à-dire une valeur très comparable à celle qu'on peut déduire (0,24 pour cent) des analyses globales de NIEMIERKO *et al.* (1956), sur des oeufs en diapause. On peut considérer que les différences individuelles notées pour les ovocytes successifs sont significatives, car elles sont en moyenne quatre fois plus fortes que celles pouvant être induites par les erreurs de dosage ainsi que le montre le calcul.

DISCUSSION

Si, indépendamment de l'allure générale des courbes, nous considérons la *variabilité individuelle* des divers caractères, nous pouvons, en nous limitant à un groupe d'ovocytes voisins, faire quelques remarques. En particulier, nous

constatons une absence de relation entre le poids et le phosphore en pour cent du poids frais; alors que la liaison entre le poids et le phosphore total n'est pas impossible.

Bien que l'on n'ait pu jusqu'ici établir une relation nette entre le poids des oeufs et le sexe, il reste possible que la variation importante du phosphore total et du phosphore exprimé en pour cent du poids frais soit de nature à influencer les phénomènes cytologiques ultérieurs tels que ceux qui président à la détermination du sexe (animal du type *Abraxas*), ou d'une façon plus générale, soit de nature à modifier l'expression de tel ou tel caractère génétique. Les expériences suggérées par de tels problèmes restent difficiles, puisque pour analyser les oeufs, on doit nécessairement les sacrifier.

VOL. 4 1960

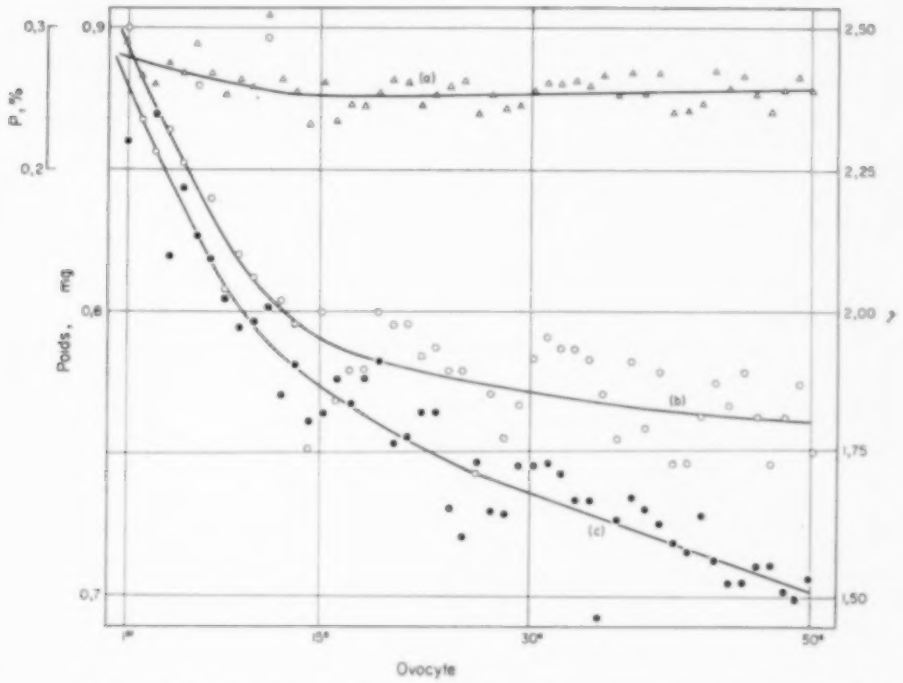


FIG. 1. (a) Variation du poids des ovocytes d'un tube ovarique suivant leur rang. (b) Variation du phosphore total des mêmes ovocytes dans le même ordre. (c) Variation de la teneur en phosphore pour cent des ovocytes correspondants. Pour chaque ovocyte: ● Poids (mg); ○ Phosphore total (γ); △ Phosphore total pour cent.

Nota—Les courbes sont tracées de façon approximative de manière à faciliter la lecture, mais n'ont pas été calculées.

Par ailleurs, il se dégage de *l'examen d'ensemble des courbes*, que le phosphore total en pour cent présent dans les ovocytes normaux de *Bombyx mori* L. se trouve être sensiblement constant; la comparaison des teneurs en phosphore des dix

premiers et des dix derniers ovocytes ne permet pas de conclure à une différence significative (test de STUDENT et FISHER: $t = 2{,}0$). Par contre, nous observons une variation générale du poids et du phosphore total des ovocytes en fonction de leur position.

En conséquence, les oeufs du premier quart de la ponte se trouvent dotés d'une quantité globale dc phosphore et de réserves vitellines nettement supérieure à celle du reste des oeufs ($t = 5{,}9$ entre les dix premiers et les dix derniers ovocytes de l'ovariole). Or, l'oeuf constituant un 'système clos' pour les corps tels que le phosphore, les embryons issus des oeufs du début de ponte possèderaient donc un avantage certain du point de vue phosphore. Cependant, au stade oeuf proprement dit, il peut apparaître une variabilité dans le métabolisme individuel, introduite par la fécondation, venant perturber l'équilibre établi dans les ovocytes entre le phosphore et l'ensemble des réserves. Un éventuel gradient lié à l'ordre des oeufs dans les tubes ovariques, serait alors modifié.

Il nous reste donc à suivre dans les mêmes conditions, la teneur relative en phosphore des oeufs après fécondation, au cours de la diapause et de l'évolution embryonnaire. Pour les mêmes raisons, nous nous sommes, d'autre part, demandés si le taux de phosphore total des ovocytes variait dans diverses conditions expérimentales, en particulier selon l'alimentation des parents, ce qui fera l'objet d'un prochain travail.

En résumé, nous observons:

(1) une grande variabilité des mesures pour les quinze premiers ovocytes de chaque ovariole,

(2) au-delà de cette zone, une variation individuelle de la teneur en phosphore total des ovocytes qui est de l'ordre de quinze pour cent.

RÉFÉRENCES

DELSAL J. L. et MANHOURI H. (1955) Étude comparative des dosages colorimétriques du phosphore. *Bull Soc. Chim. biol., Paris* **38**, 1041–1054.

LEGAY J. M. (1958) Variabilité pondérale des ovocytes chez *Bombyx mori* L. *Ann. Epiphyt.* **1**, 5–10.

NIEMIERKO P., WLODAWER P., and WOJTCZAK A. F. (1956) Lipid and phosphorus metabolism during growth of the silkworm (*Bombyx mori* L.). *Acta Biol. exp., Varsovie* **17**, 255–276.

SECRETAIN C. et SCHENK A. (1942) Nouvelle contribution à la recherche du dimorphisme ovulaire. *Recherches Séricicoles, Alès* **6**, 21–39.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 107 *to* 121. *Pergamon Press Ltd., London. Printed in Great Britain*

THE FUNCTIONS OF THE SALIVARY GLANDS OF *APIS MELLIFERA*

J. SIMPSON

Bee Department, Rothamsted Experimental Station, Harpenden, Herts.

(*Received* 5 *May* 1959)

Abstract—Worker bees dissolve dry sugar, clean their queen's body, and possibly also soften or lubricate materials being chewed, with the watery secretion of their labial glands. The small amount of oil produced by these glands accumulates in them and what little is discharged adheres to the tongue hairs and is not mixed with the food; its function is obscure. The secretion of the mandibular glands is discharged by a movement of the hypopharyngeal plate. It is an aqueous emulsion with a non-aqueous phase insoluble in lipoid solvents but soluble in alcohol, glycol, etc. The function of the secretion of workers' mandibular glands is discussed; it is not used in chewing. Salivary invertase comes only from the hypopharyngeal glands.

VOL.
4
1960

INTRODUCTION

THE morphology of the glands discharging secretions on the mouthparts of the honey bee has been described by numerous workers (RIBBANDS, 1953; SNODGRASS, 1956): little is known of their function, except for the hypopharyngeal glands.* Information about the function of the labial and mandibular glands is now presented. Anatomical nomenclature is taken from SNODGRASS (1956).

METHODS

Fresh material was dissected under distilled water, and parts of bees preserved in Pampel's fluid were cut cleanly across with scissors to reveal structural relationships.

Fatty substances were detected with solutions of Sudan III or Sudan IV in ethylene glycol; fat globules can be left in this solvent for long periods without swelling or dissolving. When the mandibular glands were to be examined the bees were anaesthetized with carbon dioxide to prevent discharge of the gland contents before their heads were removed. Staining and solubility tests of gland contents were made on a microscope slide by irrigation or by using a coverslip coated with dry stain to crush the gland and release its contents.

Amounts of invertase in glands and saliva were determined by incubating with sucrose solution buffered to pH 6·5 and then estimating glucose by the iodine oxidation method. Calibrations were made with a standard glucose solution. Control titrations were made of the test material before incubation, and of buffered sucrose before and after incubation.

OBSERVATIONS

A. Examination of the Salivary Glands and their Contents

1. *The labial glands*

In the worker bee two pairs of glands discharge through a common duct opening on the prementum between the bases of the paraglossae. One pair, the post-cerebral glands, is situated in the head and the other, the thoracic glands, in the

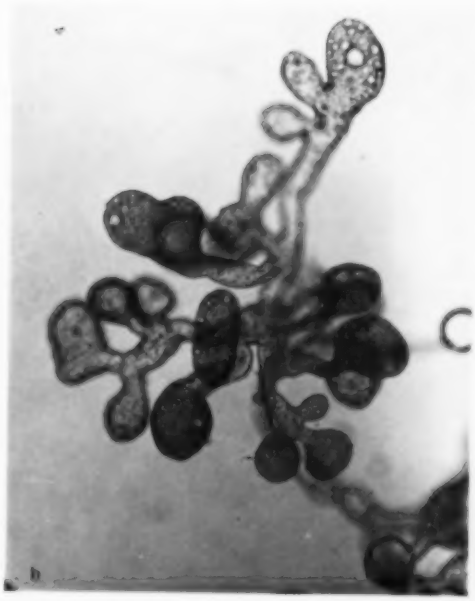
* Formerly known as 'pharyngeal' glands (see SNODGRASS, 1956).

thorax. The ducts of all four glands unite in the head and have internal spiral thickenings, resembling those in tracheae, throughout their length. At its outlet the common duct is enlarged to form a boat-shaped chamber, with stiff but elastic walls, and associated muscles, which appears to function as a pump (SNODGRASS, 1925). A single thoracic gland consists of a number of narrow tubules with relatively thick walls discharging into a thin-walled reservoir. No separate reservoir is present in the post-cerebral glands, which are made up of numerous lobes in each of which the secretory cells surround a storage cavity. The spiral thickening of the ducts presumably prevents them from collapsing when the secretion is sucked from the glands. No muscles or valves which could allow secretion to be drawn from one pair of glands independently of the other were found in the glands or ducts.

Both the reservoirs of the thoracic and lobes of the post-cerebral glands contain a clear fluid which is miscible with water and dissolves water-soluble stains. A clear oily liquid is also usually present and was observed by soaking freshly dissected glands for a few hours in Sudan IV solution. This oily material is very soluble in chloroform, ether or petroleum ether, insoluble in water or ethylene glycol, and only very slightly soluble in ethyl alcohol or acetic acid; the last solvent extracted the Sudan IV. The oil is insoluble in sodium hydroxide solution, which produced a wrinkled skin on the globules after 24 hr; concentrated hydrochloric acid produced a similar appearance within a few minutes. Heating glands crushed in ethylene glycol on a slide showed that the boiling point of the oil is above 197·5°C. Oil in the abdominal fat-bodies of worker bees has properties similar to that in the labial glands; a similar oil also occurs in the post-cerebral and thoracic glands of workers of *Apis indica* and *A. dorsata*, in the post-cerebral glands of both sexes of *Bombus spp.*, and in the thoracic glands of females of *Andrena* and *Megachile*. The sting poison sac of an *Apis indica* worker regularly contains a large globule of oil, which, however, is readily soluble in ethyl alcohol or acetic acid.

The amount of oil in honeybee labial glands varies greatly; there are usually only traces in the thoracic glands, though occasional bees are found in which all the secretory tubules and the reservoir are filled. More frequently, a few tubules contain much oil while the others have little or none. According to REIMANN (1953) only a few tubules secrete watery saliva at any one time. The post-cerebral glands frequently contain much oil, particularly after the retrogression of the hypopharyngeal glands, and the lobes are often considerably distended. (Compare a, b and c, Fig. 1.) Distended post-cerebral glands are always filled with oil and contain only a small proportion of the watery secretion. When only small quantities of oil are present, they adhere to the walls of the reservoirs and do not float freely in the watery secretion. When the glands are full of oil, the watery secretion forms spherical globules (Fig. 1, b and c) freely suspended in the oil. Thus the walls are wetted by the oil but not by the watery secretion. Oil was often found in parts of the common duct of the glands even when the duct was removed together with the tongue before the head was opened. When a gland was squeezed under a coverslip, however, the watery secretion passed through the duct without driving

VOL.
4
1960



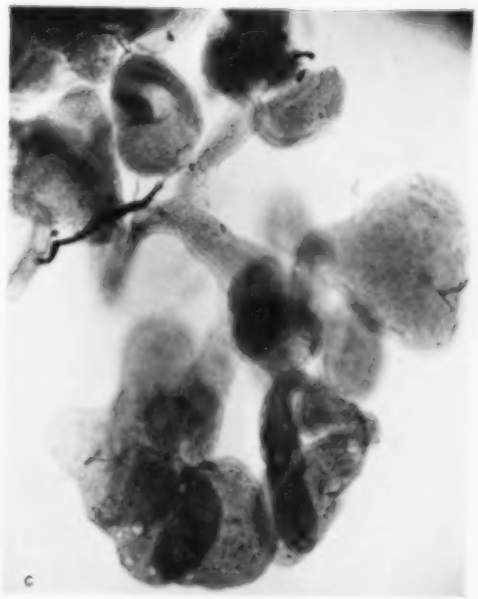


FIG. 1. Parts of post-cerebral glands of worker bees, ×100. Stained in Sudan IV and illuminated with light of complementary colour. (a) no oil. (b) filled with oil but not swollen. (c) filled with oil and swollen. Spherical globules of watery secretion can be seen among the oil in the lobes.

out the oil in front of it; while the watery secretion was passing through, the oil formed a thin layer along the walls of the duct. Secretion from the orifice of the labial glands was collected by holding a bee's tongue out with forceps and folding back the maxillae. No oil was found in secretion collected in glass capillary tubes even though the post-cerebral glands were distended with it. When the secretion was collected by absorption in filter paper impregnated with Sudan IV very little evidence of elution of the dye was observed and no translucent spot remained on drying, whereas crushing the post-cerebral glands of the same bees on Sudan IV paper produced a colourless translucent spot. The secretion collected from the gland orifice had a refractive index equal to that of 0·5 per cent sucrose solution, i.e. close to that of water. When it was evaporated, little residue remained and no charring occurred with further heating. Nevertheless some bees have oil among the hairs of their tongues, and, when the tongue of a live bee was dipped in an acetone solution of Sudan IV, the stained oil was replaced by fresh oil free from stain within a few hours.

The amount of oil in the labial glands was assessed on an arbitrary scale, grade 0 indicating absence of oil and grade 4 that the gland was full of oil (Table 1).

VOL. 4 1960

TABLE 1—OIL IN LABIAL GLANDS. NUMBERS OF BEES IN EACH GRADE

Category of bees	Date of sampling	Post-cerebral glands (grade)					Thoracic glands (grade)				
		0	1	2	3	4	0	1	2	3	4
Newly emerged	9.iv.54	6	4	0	0	0					
Newly emerged	14.v.56	6	10	4	0	0					
1 day old	15.v.56	4	13	3	0	0					
3 days old	17.v.56	1	5	9	4	1					
Overwintered, 9 months old	14.v.56	0	0	12	23	17					
Taken at random from the hive	19.v.54	66	26	14	9	15	62	43	18	6	1
	24.vi.54	68	37	22	23	20	93	56	24	6	1
	20.vii.54	49	37	21	18	25	85	54	17	3	1
	27.viii.54	57	44	16	13	20	90	23	23	10	4
	13.x.54	81	59	11	1	18	148	17	5	0	0
Foraging for pollen	19.v.54	14	5	26	35	40	21	59	39	1	0
	24.vi.54	26	26	39	41	28	48	81	25	3	3
	20.vii.54	17	25	36	32	60	23	92	46	6	3
	27.viii.54	7	9	33	39	72	61	41	78	6	4
	13.x.54	2	5	3	13	117	112	28	9	1	0
Attending queen	4.vi.54	2	2	1	0	5	2	7	0	1	0
Building comb	4.viii.54	2	2	1	1	2	3	2	3	0	0
Hypopharyngeal glands fully developed	v–x.54	234	175	80	49	47	327	160	66	6	2
Hypopharyngeal glands completely shrunken	v–x.54	31	21	54	66	161	142	105	65	9	5

Old bees are much more likely than young ones to have much oil in their post-cerebral glands, and abundance of oil in these glands can also be correlated with other changes which accompany increase in age, i.e. transfer to foraging duties and retrogression of hypopharyngeal glands. Among foraging bees the proportion with oil-filled post-cerebral glands increased greatly in August and October, perhaps indicating that only the oldest bees foraged during this period when little nectar or pollen was available. The thoracic glands showed little evidence of change with age or occupation, but abundance of oil was somewhat more frequent in August than in other summer months. According to OHNESORGE-HUMPERDINCK (1953), bees taken from colonies during the winter have very little oil in their post-cerebral glands.

The pH of the labial gland contents is difficult to measure because the secretion is poorly buffered and difficult to separate from the gland tissue. Determinations were made by adjusting indicator solutions to various levels of pH and noting in which direction colour changes, if any, were produced by the test material. Parallel tests were made with distilled water to verify that mere dilution did not cause changes in colour. Tests with crushed glands suggested a pH of 6·5–7·0 for post-cerebral glands and 6·0–6·5 for thoracic glands (KRATKY, 1931, obtained similar results). However, secretion collected from the gland orifice, and therefore free from tissue, was slightly more alkaline (ca. pH 7·5).

Queens' labial glands and their contents are similar to those of workers (no pH tests were made on them). Drones have smaller thoracic glands than workers and their post-cerebral glands consist only of a series of branched ducts, often containing oil, surrounded by cells containing fat, though, according to KRATKY (1931), a few secretory lobes usually occur among the fat cells.

2. *The mandibular glands*

A mandibular gland is a simple sac with a cuticular lining surrounded by a layer of large secretory cells. The glands are very large in queens, of moderate size in workers, and very small and possibly non-functional in drones. The orifice of a worker's gland is in the soft membrane connecting the mandible with the head. It is very close to the base of the mandible and near the edge of the clypeus above the corner of the labrum. The terminal portion of the duct is stiff, flattened and transversely curved so that its outer corners curl upwards away from the base of the mandible. Depressing the floor of the hypopharynx with a probe caused the mouth of the gland to open and release the secretion. The upper lip of the opening was pulled away from the lower, reversing its curvature so that a lenticular opening (ca. $70 \times 30\,\mu$) appeared. Examination failed to reveal any direct muscular mechanism for opening the gland, nor was it possible to cause it to open by applying tension to the apodemes of the mandibular muscles either singly or together except by dislocating the mandible. Contrary to the statement of KRATKY (1931), a gland mouth does not open automatically when the corresponding mandible is abducted, because the membrane surrounding it is too close to the clypeal articulation of the mandible to be stretched by movement of the latter. The orientation of the opening

shows that the tension required to open it must come from the direction of the hypopharyngeal plate. A fibrous band connects the corner of this plate with the upper lip of the gland orifice (Fig. 2). It appears to be merely a thickening of the

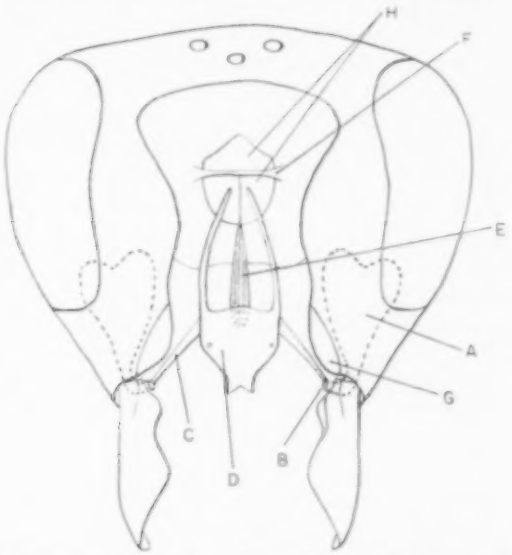


FIG. 2. Semi-diagrammatic drawing of opening mechanism of worker mandibular glands. Only the operative parts and a general outline of the head are shown. A, mandibular gland; B, outlet of gland; C, fibrous band; D, hypopharyngeal plate; E, muscle; F, tentorial bridge; G, part of clypeus bearing articulation of mandible; H, occipital foramen.

edge of the deep fold in the soft membrane at the corner of the bee's mouth, but towards the orifice of the gland the membrane is folded and elastic and therefore easily stretched to allow the fibrous band to pull on the lip of the gland. The hypopharyngeal plate is moved in the direction required to open the gland by a muscle which connects it with the tentorial bridge (SNODGRASS, 1956, Fig. 17, muscle 37). Pulling this muscle with forceps discharged the secretion. The contents of the gland are evidently under pressure, apparently because the gland wall is elastic; no muscles which could compress the gland have been found, and even in the severed head of a freshly killed bee the secretion flowed out when the outlet was opened.

When the orifice of the glands were opened in a bee with its mandibles fully separated, the secretion passed through openings between the bases of the mandibles and the edges of the clypeus and so out on to the face of the bee. With the mandibles closed, it was directed against the inside of the corners of the clypeus and diverted towards the corners of the mouth. The secretion cannot, however, be taken into the mouth along with liquid food because the mandibular gland orifices lie outside the closed tube which is formed by the lacineal lobes of the maxillae and the epipharynx when liquids are being taken up.

VOL. 4 1960

SNODGRASS (1956) suggests that the secretion flows down a groove leading from the gland orifices to the biting surfaces of the mandibles. Although there is a groove in each mandible extending upwards from the biting surface, it does not extend to the gland orifice in the way shown in Snodgrass's drawing but becomes shallower, alters its direction, and finally flattens out altogether. When the mandibles are fully folded against the epipharynx this groove may allow secretion to pass from the corner of the mouth to the biting surface, but it cannot collect it directly from the gland orifice. The mandibles are so shaped that they fit closely to the edge of the labrum when their biting surfaces are in contact. The secretion may, therefore, be conveyed to the biting surfaces by the rubbing of mandible against labrum. The openings of the mandibular glands of queens and drones are in the same position as those of workers. The opening mechanism in drones was not examined, but in queens depressing the floor of the hypopharynx discharges secretion just as with workers.

The secretion of the glands of workers is an emulsion in which one phase (phase I) is miscible with water and the other (phase II) not. Phase II is dispersed in small globules in phase I. When exposed to air, phase I evaporated leaving phase II as a clear liquid. When water was added the emulsion re-formed, suggesting that phase I is merely water. Phase II is soluble in alcohol, ethylene glycol, and 60 per cent sucrose solution but not in water, ether, benzene, or a light paraffin (b.p. about 210°C). It dissolved Sudan IV but also gave a blue solution with Nile Blue Sulphate and not the faint pink colour which this stain produces with many fats. When a mixture of these two stains was used, a few droplets were observed which stained only with Sudan IV and had solubilities similar to those of the oil in the labial glands. Phase II globules dissolved in sodium bicarbonate solution and reprecipitated with dilute hydrochloric acid (concentrated hydrochloric acid broke them up and dissolved them slowly). They were not dissolved or coagulated by trichloracetic acid and gave no precipitate with silver nitrate. They did not appear to dissolve a film of beeswax deposited on a coverslip from chloroform solution. No visible change occurred in phase II when it was heated in paraffin up to 210°C, and it still remained liquid when cooled again to room temperature. When heated dry it charred without boiling. When bees were kept at about −6°C for a few days, phase II crystallized. In an incubator in which the temperature was slowly raised from 20°C it reliquefied over the range 28–35°C. Tests of the secretion as a whole with B.D.H. 'capillator' indicators showed a pH of about 3·5–4·5. The wide-range 4–11 indicator mixture gave similar results showing that the secretion is quite well buffered. Phase II globules were stained orange yellow (i.e. pH < 6·0) by bromothymol blue applied as a layer of dry crystals on a coverslip. In old bees the emulsion is partially broken down with the formation of large colourless globules of phase II and the secretion has a strong 'cheesy-fruity' smell. In bees with large hypopharyngeal glands, the mandibular glands often contain only a watery (though acid) secretion and have a particularly thick epithelium which is easily punctured during dissection. According to KRATKY (1931) the secretory cells decrease in size as the bees get older. The secretion of queens' glands was not

tested with sucrose solution, ether, benzene, trichloracetic acid, silver nitrate, or beeswax. It is acid but its pH was not determined exactly. The large globules of phase II in old queens have a brownish colour and no smell was detected in the glands of any of the twenty-seven queens (eighteen not yet laying and nine laying) which were examined. Queen secretion did not crystallize under the conditions in which worker secretion did. In all the other tests queen and worker secretions gave very similar results.

When mandibular gland secretion was put on the abdomen of a bee and phase I evaporated, phase II remained as a sharply defined shiny spot and did not tend to flow over the rest of the body surface. If the bee was then dipped in water, the emulsion re-formed and tended to form globules which separated from the body surface and were held only by the hairs.

3. *The hypopharyngeal glands*

Previous workers (see RIBBANDS, 1953) concluded that the secretion of the hypopharyngeal glands is strongly acid. As the glands have no reservoirs, and the volume of secretory tissue is very large relative to the volume of the ducts, the pH of the secretion is difficult to determine. Tests with adjusted indicators were made both with crushed glands and by soaking the glands in indicator solutions and observing the colour of various parts under a microscope. The bees were taken

VOL. 4 1960

TABLE 2—INVERTASE IN SALIVARY GLANDS

Gland	No. of observations	No. of glands used for each estimation	Mean amount (mg) of glucose per bee produced in 24 hr	Standard error of mean
Post-cerebral	1	20	0·04	—
Thoracic				
Bees from hive	1	20	0·01	—
Bees from hive	10	5	0·02	0·06
Bees from syrup feeder on hive	10	2	0·13	0·16
Foraging bees	10	2	0·38	0·22
Mandibular				
Bees from hive	1	20	0·14	—
Bees from hive	10	5	0·62	0·20
Bees from syrup feeder on hive	10	2	−0·04	0·17
Foraging bees	10	2	0·61	0·11
Hypopharyngeal				
Newly emerged bees	11	2	0·18	0·07
Bees from hive	1	20	1·86	—
Bees from hive	20	2	1·55	0·87
Bees from hive	50	2	3·26	0·70
Bees from syrup feeder on hive	10	2	13·26	1·41
Foraging bees	12	2	3·65	0·63
Foraging bees	12	2	5·22	1·18

directly from a hive and their age and occupation were unknown. The results suggested that although the outside of the secretory lobes is acid (ca. pH 5·0) the secretion is less so (ca. pH 6·0).

4. *Invertase in the glands*

For these tests the glands were ground to release the enzyme. Substantial though variable quantities of invertase were found in the hypopharyngeal glands but little, if any, in the post-cerebral, thoracic, or mandibular glands (Table 2). The standard errors associated with the smallest means may be taken to indicate the errors involved in the method of measurement, the larger standard errors for the larger means being due to the great variation between individual bees.

B. The Saliva Discharged by Bees under Various Circumstances

1. *Saliva used to dissolve dry sugar*

Worker bees can be fed for several days on finely powdered dry sucrose provided they are also supplied with water. Microscopic observation showed that the sugar is taken up with the tongue and not with the mandibles and that saliva is discharged to dissolve it. Only the tongue appeared wet; the outside of the maxillae became encrusted with dry sugar. For closer examination bees were held by the neck between V-shaped pieces of black cardboard (MARSHALL, 1935) and allowed to take up dry sugar from the end of a small glass tube, which they would usually do after the taste organs in their antennae were stimulated with sugar syrup. When a bee tried to feed on the sugar with its maxillae held back, the saliva did not come from the mouth but appeared at the edges of the paraglossae, which conceal the orifice of the labial glands, and flowed down the tongue among its hairs. The course of the saliva down the tongue was followed by putting a particle of Light Green stain between the paraglossae; it seemed that the movement of the tongue with its distally directed hairs against the labial palps propelled the saliva rapidly down the back of the tongue. SNODGRASS (1956) suggests that saliva can only be discharged when the tongue is extended because the muscles, which are attached to the edge of the salivary pump and which he thinks responsible for flattening it after it has been dilated by the muscles attached to its dorsal surface, are slack when the base of the tongue is retracted into the prementum. The pump, however, tends to flatten by its own elasticity; this can be seen by dissecting it out of a newly killed bee. Thus it may perhaps be able to function without the help of the muscles attached to its edge. Saliva discharged when the tongue is retracted and the outlet of the pump is enclosed by the fold of the prementum could pass out through the channels formed by the incurved paraglossae.

The pH of the saliva discharged during feeding on sugar was tested with indicators previously adjusted to various pH levels. The sugar was placed on filter paper so that the solution was absorbed before it could be taken up by the bees. After part of the sugar had been consumed, a drop of distilled water was added to dissolve the residue and extract the salivary material from the paper. The resultant

solution was tested with the adjusted indicators. Bees were also allowed to feed on sugar placed on filter paper previously soaked in adjusted indicators and dried. These tests suggested that the saliva has a pH of about 7·2–8·0 but is too weakly buffered for accurate determination with indicators.

The surface layer of sucrose candy partially consumed by bees was dissolved in Sudan III solution, but no globules of oil were found. However, when bees were allowed to consume dry sugar from a microscope slide, treatment with Sudan III revealed traces of oil on the slide in fourteen trials out of thirty-two. A further test was made by putting bees in a cage which was placed on a sheet of filter paper lying on a sheet of glass. A hole in the bottom of the cage allowed the bees access to a limited area of the paper which was covered with finely powdered Analar sucrose. A few hours' feeding produced a wet spot which remained translucent after drying. Soaking the paper in water to remove the sugar and re-drying it destroyed the translucency, and treatment with Sudan IV solution showed no oily residue.

VOL. 4 1960

The filter paper method was also used to collect saliva for invertase tests. For each test twenty bees were caged and allowed to feed on about 1 g sucrose. The wet patch in the paper was cut out and soaked in 3 cm^3 of buffer solution. The amount of glucose in 1 cm^3 of the resultant solution was estimated before and after incubating for 24 hr. Control tests were made with clean filter paper. The results showed a mean production of 0·19 mg of glucose per bee (s.e. 0·05 mg, 46 d.f.). The results of the individual tests ranged from −0·06 mg to 0·97 mg per bee (s.e. 0·09 mg, 19 d.f.—based on the variance of the controls). Evidently the saliva deposited on the sugar contained variable amounts of invertase though there is no basis for comparing these amounts with those to be expected if some saliva came from the hypopharyngeal glands.

Bees which had been fed on syrup strongly stained with Light Green were allowed to feed on dry sugar on filter paper. One bee was put into each cage with the sugar. Although the bees made a few green marks on the paper they also produced colourless translucent spots, thus confirming that the saliva used to dissolve the sugar was not merely material regurgitated from the honey stomach.

2. *Saliva used in chewing filter paper*

Any soft material put among the bees of a colony is chewed to shreds and removed from the nest. When a sheet of filter paper was pinned to the top bar of a comb frame and left until it had been thoroughly shredded at the edges, these and adjacent parts of the paper were damp. The pH of this saliva was tested in two ways: (1) by soaking chewed paper in distilled water and testing the extract with indicators, and (2) by allowing the bees to chew indicator papers. The saliva was always alkaline, pH 7·2–8·5. On one occasion Thymol Blue was turned blue (i.e. pH > 9·0) by the extract from a chewed paper but this could not be repeated. Damp paper suspended among the bees but protected from chewing showed no sign of alkalinity, so the saliva must be alkaline when discharged and not made so by the hive atmosphere. When bees in an observation hive chewed filter paper

stained with the water soluble stain Light Green, their saliva eluted the stain when it flowed out into the paper. Sudan IV was not eluted. No blue colour appeared on paper which was chewed by a colony in which the mandibular gland contents of the bees were stained with Nile Blue Sulphate (see below) and no green colour resulted from similar chewing by a colony fed on syrup strongly coloured with Light Green. Chewed paper showed no sign of oil when treated with Sudan IV solution and only sporadic spots of cuprous oxide appeared when it was boiled in Fehling's solution.

3. *Saliva used by worker bees when licking their queen*

A comb bearing brood, honey, worker bees and a queen was placed in an observation chamber at 34°C. The queen was tethered on the comb with a piece of stiff wire so that she remained in the field of a microscope, and concentrated solutions of stains were painted on her body and allowed to dry. The worker bees immediately began to lick the queen. With Light Green, which in the solid state is dark and lacks colour, a bright green solution immediately bathed the bees' tongues. It was rapidly removed from the queen's body and could then be seen through the abdominal wall, in the worker bees' honey stomachs. With Sudan IV, which is equally lacking in colour when dry, no sign of solution was observed, though colourless saliva could be seen on the bees' tongues; the dye appeared to be removed only by slow abrasion.

4. *Saliva used in working wax*

No opportunity occurred for observing bees building comb. Young bees cutting their way out of the cells in which they had pupated were seen to have saliva on their mandibles. This saliva did not have the milky appearance of mandibular gland secretion. ÖRÖSI-PÁL (1957) claims that when Nile Blue Sulphate dissolved in sugar syrup is fed to bees, the contents of their mandibular glands become blue and any new comb built is also blue. According to this author the wax scales in the wax glands of bees fed with Nile Blue Sulphate are not coloured, whereas feeding with Sudan III (in oil solution mixed with sugar candy) produces coloured wax scales as well as coloured combs. Attempts were made to verify these observations. Feeding Sudan III to bees in a cage did make the scales in the wax glands red but it also coloured the whole surface of the bees' bodies, the inside of the cage, and a piece of comb in the cage. Thus the bees seem unable to handle the oily mixture cleanly, and their wax scales were probably coloured by external contamination. Contamination of this kind was not observed when Nile Blue Sulphate was fed, and the contents of many of the bees' mandibular glands became coloured without any blue appearing in their wax scales. This dye, in the concentration (0·05 per cent) prescribed by ÖRÖSI-PÁL, was fed in sugar syrup to a colony of bees until the colour appeared in their mandibular glands, but the new comb built by the colony was white except in the cells in which coloured syrup was stored.

DISCUSSION

1. *Saliva*

The foregoing observations show that the saliva used by worker bees to dissolve dry sugar comes mainly from the labial glands. The invertase in it probably comes from contamination of the tongue with honey stomach contents during food exchange (NIXON and RIBBANDS, 1952; FREE, 1957). Direct supply of the enzyme to the tongue by the hypopharyngeal glands seems to be impossible because the orifices of these glands lie within the ingoing food stream during feeding. Saliva with a refractive index equivalent to about 15 per cent sucrose solution, and therefore not from the labial glands alone, is added to food in the honey stomach (PASEDACH-POEVERLEIN, 1940).

The saliva used by worker bees when cleaning their queen's body probably also comes from the labial glands, because it is discharged with the tongue extended. The absence of a lipoid solvent in it rules out the mandibular glands as a possible source.

The source of the saliva used by bees when chewing paper is less certain. It cannot come from the hypopharyngeal glands, mandibular glands, or honey stomach, but its alkalinity seems to be greater than that of labial gland secretion.

VOL. 4 1960

2. *Labial glands*

KRATKY (1931) found no invertase, diastase, or protease in the labial glands, whereas INGLESENT (1940) concluded that the thoracic part of these glands is the principal source of salivary invertase. Neither the present results nor those of KOSMIN and KOMAROV (1932) support INGLESENT'S conclusion.

The secretion of the labial glands consists of two clearly distinguishable portions, a watery phase, perhaps nearly pure water, used to soften or dissolve food and other materials, and an oily phase, of which the functions, if any, are obscure. HESELHAUS (1922) suggested that the oil is used to soften wax during its manipulation, but similar oil occurs in the labial glands of bees that do not produce wax. INGLESENT (1940) found that addition of post-cerebral gland contents lowered the melting point of wax, but this effect could have been produced by adding any fat of low melting point.

The apparent absence of any means by which the oil can be separately discharged, and the ability of the watery secretion to pass through the ducts without carrying the oil along with it, suggest that little oil is discharged except when it has accumulated to such an extent that it overflows from the glands. The amount of oil produced must be small in proportion to the amount of watery secretion, because little oil is discharged even by bees with post-cerebral glands distended with oil. The similarity between the properties of labial gland oil and fat body oil, and the production of oil by the sting glands of *Apis indica*, suggests that oil is an unimportant by-product of certain kinds of glandular tissue in bees, perhaps produced during degeneration of the secretory cells. However, the adhesion to the tongue hairs of any oil discharged with the saliva suggests that the oil may have some other function. From the tongue it may be transferred to the legs when the tongue is

cleaned and thence to other parts of the body by routine cleaning movements (BEECKEN, 1934). Possibly it helps to maintain the properties of cuticle; if so, old bees whose duties involve flying may need most oil.

3. *Hypopharyngeal glands*

In addition to invertase the hypopharyngeal glands produce at least a major part of the food given to larvae (evidence reviewed by RIBBANDS, 1953). Although it is not certain that both cannot be produced simultaneously, invertase production is probably greatest when the lobes of the glands have at least partially retrogressed from the large size which is associated with feeding brood (KOSMIN and KOMAROV, 1932; BUTLER and SIMPSON, 1951). The hypopharyngeal glands also produce an enzyme that facilitates the oxidation of glucose to an acid, which ensures the invariable acidity of honey (GAUHE, 1940). From the absence of any storage reservoirs, closing mechanisms, or internal thickenings in the glands or their ducts, it seems that the secretion must be discharged as fast as it is produced, but as the ducts open on the hypopharyngeal plate, it is difficult to see how food for larvae is kept separate from food being ingested by nurse bees for their own use.

INGLESENT (1940) claimed to have shown that these glands secrete a protease, but consideration of his method and results suggests that he may merely have been demonstrating the occurrence of soluble protein in the glands. Neither KRATKY (1931), HITCHCOCK (1956), nor BUTLER (personal communication) found a protease in them.

4. *Mandibular glands*

The mandibular glands of queens produce the substance responsible for inhibiting queen production by colonies which already have queens (BUTLER and SIMPSON, 1958). The worker bees collecting this substance lick all parts of their queen's body surface (BUTLER, 1954) so it would seem that the contents of the mandibular glands of a queen must be spread over her body when she cleans herself. If they have properties similar to those of the secretion from workers' glands, they will readily be removed when the workers wash the queen with watery saliva from their labial glands. The cleaning movements of worker bees are similar to those of queens (BEECKEN, 1934), so the spreading of the secretion over the body surface may have some general function common to both workers and queens—but perhaps not to drones in which these glands are rudimentary. Certain other species of bees use mandibular gland secretion, deposited by the mandibles, as a sexual attractant (KULLENBERG, 1956) and for marking routes to food sources (LINDAUER and KERR, 1958). The scented substance in the glands of worker honeybees may also have a communicative function, but so far none has been demonstrated.

An important function of the mandibles is the chewing of wax and other soft material but the present evidence does not suggest that the mandibular glands are involved in this. They do not provide the saliva used in chewing filter paper, and the results of feeding Nile Blue Sulphate to colonies did not support the hypothesis

that the secretion is added to wax. According to LINEBURG (1924a) the fluid on the mandibles of bees which are chewing wax scales is neutral or slightly alkaline to litmus; if so, it cannot come from the mandibular glands and may have the same source as the saliva used in chewing filter paper. The mandibles are also used for giving food to larvae (LINEBURG, 1924b). When doing this they vibrate, which presumably results in the flow of food down the mandibles from the mouth.* No conclusion about the source of saliva seen on the mandibles can be drawn from the positions of the gland openings, as these are all in a position to discharge into the mouth when the tongue is folded.

SUMMARY

(1) The secretion of the labial (post-cerebral and thoracic) glands is nearly neutral and weakly buffered and consists of two quite separate liquid phases. One is a clear watery fluid almost free from solutes, the other a clear oil with properties resembling those of the oil in the abdominal fat-bodies. Similar oil occurs in other organs of the honeybee and in other social and solitary bees. The thoracic glands rarely contain much oil but the post-cerebral glands are often filled and distended with it, particularly in bees in which the hypopharyngeal glands have retrogressed.

(2) Secretion discharged from the common orifice of the labial glands contains little oil even when the post-cerebral glands are full of it. The oil adheres to the walls of the reservoirs and ducts with the result that the watery secretion can be discharged without oil. Any oil discharged appears to be retained by the hairs of the tongue and not mixed with the food. Little oil is produced in comparison with the amount of watery secretion.

(3) The secretion of the mandibular glands is a strongly acid emulsion of an aqueous phase and a phase insoluble in water; the latter is also poorly soluble in several lipoid solvents (though it dissolves Sudan IV stain) but is readily soluble in alcohol. It dissolves in alkalies and is re-precipitated by acids.

(4) The contents of a mandibular gland are under pressure and flow out when the gland mouth is opened. Opening is not produced by movement of the mandibles but by lowering and retracting the hypopharyngeal plate by a muscle which pulls it towards the tentorial bridge.

(5) The hypopharyngeal glands are the only salivary glands which contain appreciable quantities of invertase. Their secretion is slightly acid, and probably is added to food only after it reaches the pharynx or honey stomach.

(6) Worker bees feeding on dry sugar dissolve it in saliva from their labial glands. Invertase detectable in food wet with saliva may come from contamination of the mouthparts with honey stomach contents during food exchange.

* After this paper was submitted for publication it was shown (CALLOW *et al.*, 1959; BARKER *et al.*, 1959b) that the crystallizable material in workers' mandibular glands is 10-Hydroxy-Δ^2-decenoic acid, the main lipoid component of the food of queen and worker larvae (BUTENANDT and REMBOLD, 1957; BARKER *et al.*, 1959a). The appearance of the hypopharyngeal glands when treated with mandibular gland secretion had already led HAYDAK (1957) to infer the presence of the latter in larval food.

VOL. 4 1960

(7) When worker bees chew paper they moisten it with alkaline saliva from an obscure source.

(8) When cleaning their queen's body, worker bees use saliva which dissolves water-soluble but not fat-soluble stains and probably comes from the labial glands.

(9) Feeding Nile Blue Sulphate to worker bees colours the contents of their mandibular glands but does not result in the production of coloured combs. If bees use any saliva when working wax, it probably comes from the same source as the saliva used in chewing paper and not from the mandibular glands.

(10) Possible functions of the oil from the labial glands and the secretion of the mandibular glands are discussed.

REFERENCES

BARKER S. A., FOSTER A. B., LAMB D. C., and HODGSON N. (1959a) Identification of 10-hydroxy-Δ^2-decenoic acid in royal jelly. *Nature, Lond.* **183**, 996–997.

BARKER S. A., FOSTER A. B., LAMB D. C., and JACKMAN L. M. (1959b) The biological origin and configuration of 10-hydroxy-Δ^2-decenoic acid. *Nature, Lond.* **184**, 634.

BEECKEN W. (1934) Ueber die Putz- und Sauberungshandlungen der Honigbiene (*Apis mellifica*). *Arch. Bienenk.* **15**, 213–275.

BUTENANDT A. and REMBOLD H. (1957) Ueber die Weiselzellenfuttersaft der Honigbiene —I. Isolierung, Konstitutionsermittlung und Vorkommen der 10-Hydroxy-Δ^2-decensäure. *Hoppe-Seyl. Z.* **308**, 284–289.

BUTLER C. G. (1954) The method and importance of the recognition by a colony of honeybees (*A. mellifera*) of the presence of its queen. *Trans. R. ent. Soc. Lond.* **105**, 11–29.

BUTLER C. G. and SIMPSON J. (1951) *Rep. Rothamst. exp. Sta.* pp. 112–113.

BUTLER C. G. and SIMPSON J. (1958) The source of the queen substance of the honeybee (*Apis mellifera* L.). *Proc. R. ent. Soc. Lond.* A **33**, 120–122.

CALLOW R. K., JOHNSTON N. C., and SIMPSON J. (1959) 10-Hydroxy-Δ^2-decenoic acid in the honeybee (*Apis mellifera*), *Experientia* **15**, 421.

FREE J. B. (1957) Transmission of food between worker honeybees. *Brit. J. anim. Behav.* **5**, 41–47.

GAUHE A. (1940) Ueber ein glukoseoxydierendes Enzym in der Pharynxdrüse der Honigbiene. *Z. vergl. Physiol.* **28**, 211–253.

HAYDAK M. H. (1957) Changes with age in the appearance of some internal organs of the honeybee. *Bee World* **38**, 197–207.

HESELHAUS F. (1922) Die Hautdrüsen der Apiden und verwandter Formen. *Zool. Jb. (Anat.)* **43**, 369–464.

HITCHCOCK J. D. (1956) A milk-digesting enzyme in pollen stored by honeybees. *Amer. Bee J.* **96** (12), 487–489.

INGLESENT H. (1940) Zymotic function of the pharyngeal, thoracic and post-cerebral glands of *Apis mellifica*. *Biochem. J.* **34**, 1415–1418.

KOSMIN N. P. and KOMAROV P. M. (1932) Ueber das Invertierungsvermogen der Speicheldrüsen und des Mitteldarmes von Bienen verschiedenen Alters. *Z. vergl. Physiol.* **17**, 267–279.

KRATKY E. (1931) Morphologie und Physiologie der Drüsen in Kopf und Thorax der Honigbiene (*Apis mellifica* L.) *Z. wiss. Zool.* **139**, 120–200.

KULLENBERG B. (1956) Field experiments with chemical sexual attractants on Aculeate Hymenoptera males—I. *Zool. Bidr. Uppsala* **31**, 253–354.

LINDAUER M. and KERR W. E. (1958) Die gegenseitige Verständigung bei den stachelosen Bienen. *Z. vergl. Physiol.* **41**, 405–434.

LINEBURG B. (1924a) Comb building. *Amer. Bee J.* **64**, 271–272.

LINEBURG B. (1924b) The feeding of honeybee larvae. *Bull. U.S. Dep. Agric.* **1222**, 25–37.
MARSHALL J. (1935) On the sensitivity of the chemoreceptors of the antenna and fore-tarsus of the honeybee, *Apis mellifica* L. *J. exp. Biol.* **12**, 17–26.
NIXON H. L. and RIBBANDS C. R. (1952) Food transmission within the honeybee community. *Proc. roy. Soc.* (B) **140**, 43–50.
OHNESORGE-HUMPERDINCK I. (1953) Beobachtungen an Kopfdrüsen der Honigbiene. *Naturwissenschaften* **40**, 2.
ÖRÖSI-PÁL Z. (1957) The role of the mandibular glands of the honeybee. *Bee World* **38**, 70–73.
PASEDACH-POEVERLEIN K. (1940) Ueber das 'Spritzen' der Bienen und die Konzentrationsänderung ihres Honigblaseninhalts. *Z. vergl. Physiol.* **28**, 197–210.
REIMANN K. (1953) Untersuchungen an der Thoraxdrüse der Arbeiterin von *Apis mellifica*. *Naturwissenschaften* **40**, 539.
RIBBANDS C. R. (1953) *The Behaviour and Social Life of Honeybees*. Bee Research Association, London.
SNODGRASS R. E. (1925) *Anatomy and Physiology of the Honeybee*. McGraw-Hill, New York.
SNODGRASS R. E. (1956) *Anatomy of the Honey Bee*. Cornell University Press, New York.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 122 *to* 127. *Pergamon Press Ltd., London. Printed in Great Britain*

DIPHASIC AND MONOPHASIC RESPONSES IN THE COMPOUND EYE OF *CALLIPHORA*

H. AUTRUM* and C. HOFFMANN

Zoological Institute, University of München

(*Received* 2 *June* 1959)

Abstract—The normal retinogram of the compound eye of *Calliphora erythrocephala* is diphasic and possesses a flicker fusion frequency of about 240 stim/sec. This is not decreased by light adaptation. Dark adaptation occurs very rapidly. The negative component, which originates from the retinula cells, can be isolated by lowering the partial pressure of oxygen. This component has a low fusion frequency of about 60 stim/sec, which can be strongly reduced by light adaptation. A long time is required for dark adaptation. The positive component is a prerequisite for the resolution of high flicker frequencies. The negative component cannot follow high flicker frequencies. A relationship exists between potential form and inertia of the compound eye, contrary to the assumption of RUCK (1958).

THE compound eyes of many insect species respond to illumination with a potential consisting of several phases (for summary see WULFF, 1956). The electroretinogram (e.r.g.) begins in these insects with a steep positive on-effect, which may be preceded under certain conditions by a small negative deflection. After cessation of the light stimulus, a negative off-effect follows. In previous papers it has been shown that this diphasic e.r.g. in *Calliphora*, *Eristalis*, *Apis*, *Bombus*, and other insects has a high flicker fusion frequency between 200 and 300/sec, and shows rapid adaptation (AUTRUM, 1948a, 1949, 1950, 1952; AUTRUM and STOECKER, 1950). AUTRUM and GALLWITZ (1951) were able to obtain a pure negative response from the isolated retinula of *Calliphora*, by removal of the optic ganglia. This result was independently confirmed by HARTLINE *et al.* (1952). This monophasic response can be equated with the negative potentials obtained from other insects (*Tachycines*, *Dixippus*, *Periplaneta*); these potentials have a low flicker fusion frequency and show a slow adaptation (AUTRUM, 1950, 1952; WALTHER, 1958a, b).

On the basis of these comparative investigations, it was assumed that the retinula cells alone deliver a purely negative response, and that the diphasic potential arises from the interaction of (at least) two components of opposite sign. The negative component arises in the visual cells, and the positive component in the closely adjacent ganglion cells of the lamina ganglionaris (AUTRUM, 1950, 1952). The high fusion frequency of eyes possessing diphasic potentials was attributed to the specific action of the positive component.

The work of AUTRUM and HOFFMANN (1957) made it probable that the positive component arises postsynaptically, that is, not in the visual cells themselves.

* The investigations reported here were supported by the Deutsche Forschungsgemeinschaft.

Poisoning with nicotine, a specific synaptic poison (PRINGLE, 1939; WIERSMA and SCHALLECK, 1948; KRUPP *et al.*, 1952), leads to disappearance of the positive portion of the diphasic e.r.g., whereas the monophasic negative portion remains unchanged. Nicotine has no influence upon potentials which are monophasic to begin with, as in *Dixippus* (AUTRUM, 1958).

In contrast to these results, RUCK (1958) arrived at the conclusion that no necessary relationship exists in the compound eye (or in the ocelli) between potential form on the one hand, and fusion frequency and adaptation on the other.

It was therefore of interest to investigate monophasic and diphasic responses in the same animal with regard to their fusion frequency and adaptation. This is possible when oxygen is largely withdrawn from the eye (HOFFMANN, unpublished). At an oxygen concentration of 0·1–0·2 per cent (+99·5 per cent N_2) the diphasic e.r.g. becomes purely monophasic, and the eye can be maintained in this state for at least 90 min. When oxygen is again supplied to the eye, the diphasic potential appears again.

VOL. 4 1960

METHOD

Experimental animals were females of the 'white-apricot' mutant strain of *Calliphora erythrocephala* Meig., which lack the red eye pigment of the wild form. This mutant has the advantage that the eyes are more sensitive to light as a result of the lack of protective pigment. The animals were taken from controlled stocks maintained at the München Zoological Institute. The head was removed for the experiment. The different electrode (silver–silver-chloride) rested on the outer surface of the eye; the indifferent electrode was inserted into the head through the occipital opening. The preparation was placed in a gas-tight chamber, into which a moisture-saturated mixture of 0·1 per cent O_2 and 99·9 per cent N_2 could be led at will. Potentials were registered by means of a directly-coupled amplifier and oscillograph connected with a photographic camera. Light source was a 30 W incandescent light bulb fed by stabilized direct current. Stimulus length in all experiments was 0·144 sec. Flicker light was provided by a rotating sectored disk. Stimulus traces were registered with a photoelement and therefore report only stimulus frequency, not the shape of the light stimulus.

RESULTS

(1) The normal e.r.g. of *Calliphora* is diphasic (Fig. 1a). Its fusion frequency, at the illumination intensity of the experiments is 240 stim/sec. The e.r.g. responded to flickering light after a refractory period* of about 55 msec, with steep potentials synchronous with the flicker frequency (Fig. 1b).

In other respects the e.r.g. of *Calliphora* has been thoroughly described by AUTRUM (1950). Reference is made to the data published in that paper.

(2) Under reduced oxygen pressure (0·1–0·2 per cent), the e.r.g. becomes monophasic after some time (Fig. 2); it begins with a negative deflection, which

* The duration of the refractory period is dependent on light intensity.

decreases somewhat in the first 80 msec and then remains relatively constant during the remainder of the stimulus. The fusion frequency of this negative potential sinks to 60 stim/sec (Fig. 2).

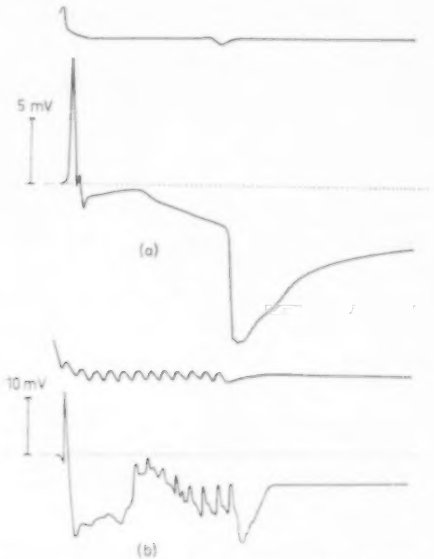


FIG. 1. *Calliphora erythrocephala*. (a) Normal, diphasic e.r.g. Constant stimulus, 0·144 sec duration. Registration begins with the beginning of illumination. Upper trace, stimulus. Lower trace, e.r.g. (59–1/1·5). (b) Flicker frequency 85/sec (59–3/1·2). Positive potentials deflected upwards.

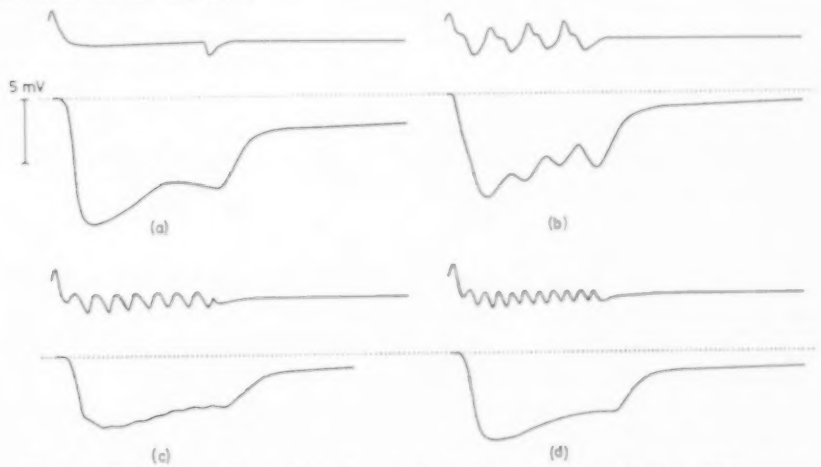


FIG. 2. *Calliphora erythrocephala*. Twenty-five minutes after introduction of 0·1 per cent O_2 and 99·9 per cent N_2; a monophasic e.r.g. remains. (a) Constant stimulus of 0·144 sec duration. (b) Flickering light, 28 stim/sec. (c) 57 stim/sec. (d) 77 stim/sec (*cf*. Fig. 1b) (59–1, 9, 10, 13, 11).

(3) The diphasic e.r.g. shows no significant (lasting longer than 1 sec) reduction in sensitivity after 1 min adaptation with a steady light source. The monophasic e.r.g. is, however, very sensitive to light adaptation. Illumination at full intensity for 1 min may cause it to disappear almost completely. It regains its original amplitude in the course of a longer period (more than 10 min) of continuous dark adaptation. As a consequence it was necessary to determine fusion frequency only within short periods of stimulation (0·144 sec each), and to introduce a pause of at least 3 min between individual periods; otherwise too low a value for fusion frequency would have been obtained. In the interests of completeness it should be mentioned that under the same stimulus conditions (0·144 sec flickering light) the fusion frequency of the diphasic potential was 240 stim/sec.

(4) While the eye is under oxygen lack, the positive component becomes smaller after some time. By admitting appropriate small amounts of oxygen, such a preparation can be maintained in a state in which a small remnant of the positive

VOL. 4 1960

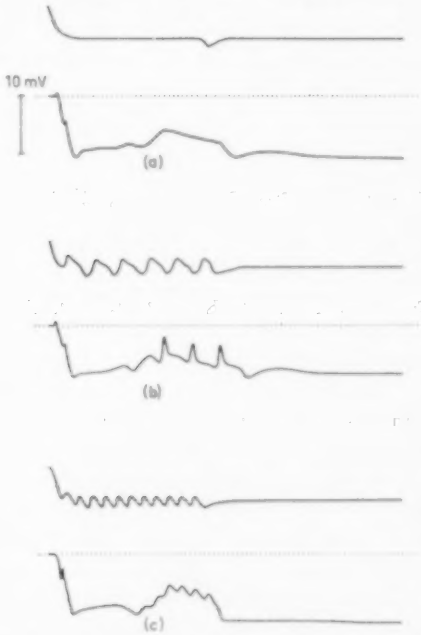


FIG. 3. *Calliphora erythrocephala*. E.r.g. with remnants of the positive component under oxygen lack. (a) Constant stimulus, 0·144 sec duration. (b) 48 stim/sec. (c) 83 stim/sec (59–3/2, 11, 10, 7).

component is still present, while greatly outweighed by the negative component (Fig. 3). This positive component follows the flicker frequency to the usual high values. At the same time the peculiar character of this positive component is manifested in the much greater steepness of the (reduced) flicker responses (Fig. 3). Following the onset of the potential there is a period in which it is refractory to

the flicker (Fig. 3). The adaptation of this potential with a small positive remnant could not be more precisely investigated, because it is difficult to regulate the oxygen supply so exactly that such potentials remain constant over a sufficiently long period of time.

DISCUSSION

The experiments of AUTRUM and GALLWITZ (1951) and AUTRUM and HOFFMANN (1957) have shown that (at least) two electrical components of opposite sign are contained in the diphasic e.r.g. of *Calliphora*. They can be separated by removal of the optic ganglia or by means of a specific synaptic poison, nicotine. The negative component, localized by AUTRUM and GALLWITZ in the retinula cells, still persists in both cases. Oxygen lack also leads to isolation of a negative component. In addition it can easily be demonstrated that uncontrolled damage to the eye leads to a conversion of the diphasic potential into a slow, monophasic potential in which small remnants of the positive component still persist. This is the case, for example, after long periods of experimentation, or when the eye has been damaged during preparation. A cause of purely negative potentials or intermediate forms with positive remnants can at times be even slight pressure on the thorax or abdomen when the animal is caught, or when the instruments used in preparation are not quite sharp, or perhaps also the pressure of aluminium foil upon the eye (the method of RUCK, 1958).

It is probable that, among others, the potential forms which RUCK (1958) gives for the bee, for example, originate from damaged preparations. Bees are very sensitive to a relatively brief (30 min) lack of nutrition. The e.r.g. of bees has been previously shown by AUTRUM and STOECKER (1950) to be diphasic, when the animals are carefully handled. The experiments reported in (4) and shown in Fig. 3 demonstrate that such a predominantly monophasic e.r.g. may still simulate a high fusion frequency when remnants of the positive component are still present. In RUCK's Figs. 5 (*Apis*), 9 (*Pachydiplax*), and 11 (*Photmia*) such positive remnants are present. At low intensities (slow increase of the negative component: $\log I = -6{\cdot}0$ and $-5{\cdot}0$; RUCK's Fig. 5) they appear as positive waves. At higher intensities (rapid increase of the negative component, $\log I = -4{\cdot}5$ and $-4{\cdot}0$, RUCK's Fig. 5) they appear as a distinctive hump at the beginning of the potential. On the basis of our experience we therefore cannot designate the potentials pictured by RUCK as normal retinograms. RUCK's statements about the bee are now being checked in our laboratory, and a report will soon be published.

The experiments on *Calliphora* show that the normal e.r.g. is diphasic and has a high fusion frequency. The negative component, which has with certainty been localized in the retinula cells, can be isolated. It has a much smaller fusion frequency. The whole course of the responses to flicker is slower; this is clearly shown by the flat waves of Fig. 2 as compared with RUCK's Fig. 4. The high fusion frequency therefore is based on the presence of the positive component. AUTRUM and HOFFMANN (1957) have shown that this positive component arises postsynaptically. AUTRUM (1950, 1952) and AUTRUM and GALLWITZ (1951) localize the positive

component in the optic ganglia and assume that the high fusion frequency of the compound eye is based on the collaboration of positive and negative components. It remains to be shown through further experiments whether this hypothesis is correct. All previous investigations—in contrast to the claims of RUCK—support the assumption that a close, lawful relationship exists between potential form, fusion frequency, inertia, and adaptation of the compound eyes of insects.

REFERENCES

AUTRUM H. (1948a) Über das zeitliche Auflösungsvermögen des Insektenauges. *Nachr. Akad. Wiss. Göttingen* **1948**, 8–12.

AUTRUM H. (1948b) Zur Analyse des zeitlichen Auflösungsvermögens des Insektenauges. *Nachr. Akad. Wiss. Göttingen* **1948**, 13–18.

AUTRUM H. (1949) Neue Versuche zum optischen Auflösungsvermögen fliegender Insekten. *Experientia* **5**, 271–277.

AUTRUM H. (1950) Die Belichtungspotentiale und das Sehen der Insekten (Untersuchungen an *Calliphora* und *Dixippus*). *Z. vergl. Physiol.* **32**, 176–227.

AUTRUM H. (1952) Über zeitliches Auflösungsvermögen und Primärvorgänge im Insektenauge. *Naturwissenschaften* **39**, 290–297.

AUTRUM H. (1958) Electrophysiological analysis of the visual systems in insects. *Exp. Cell. Res.* (*Suppl.*) **5**, 426–439.

AUTRUM H. and GALLWITZ U. (1951) Zur Analyse der Belichtungspotentiale des Insektenauges. *Z. vergl. Physiol.* **33**, 407–435.

AUTRUM H. and HOFFMANN E. (1957) Die Wirkung von Pikrotoxin und Nikotin auf das Retinogramm von Insekten. *Z. Naturf.* **12 b**, 752–757.

AUTRUM H. and STOECKER M. (1950) Die Verschmelzungsfrequenzen des Bienenauges. *Z. Naturf.* **5 b**, 38–43.

HARTLINE H. K., WAGNER H. G., and MACNICHOL E. F. (1952) The peripheral origin of nervous activity in the visual system. *Cold. Spr. Harb. Symp. quant. Biol.* **17**, 125–141.

KRUPP H., LENDLE L., and STAPENHORST K. (1952) *Arzneimittel-Forsh.* **2**, 258 (cit. from AUTRUM and HOFFMANN, 1957).

PRINGLE J. W. S. (1939) The motor mechanism of the insect leg. *J. exp. Biol.* **16**, 220–231.

RUCK P. (1958) A comparison of the electrical responses of compound eyes and dorsal ocelli in four insect species. *J. Ins. Physiol.* **2**, 261–274.

WALTHER J. B. (1958a) Untersuchungen am Belichtungspotential des Komplexauges von *Periplaneta* mit farbigen Reizen und selektiver Adaptation. *Biol. Zbl.* **77**, 63–104.

WALTHER J. B. (1958b) Changes induced in spectral sensitivity and form of retinal action potential of the cockroach eye by selective adaptation. *J. Ins. Physiol.* **2**, 142–151.

WIERSMA C. A. G. and SCHALLEK W. (1948) Influence of drugs on response of a crustacean synapse to preganglionic stimulation. *J. Neurophysiol.* **11**, 491–496.

WULFF V. J. (1956) Physiology of the compound eye. *Physiol. Rev.* **36**, 145–163.

VOL. 4 1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 128 *to* 137. *Pergamon Press Ltd., London. Printed in Great Britain*

THE EFFECT OF DDT AND OF PHYSICALLY INDUCED PROSTRATION ON ACETYLCHOLINE LEVELS IN THE COCKROACH

S. E. LEWIS, J. B. WALLER, and K. S. FOWLER

Pest Infestation Laboratory, Slough, Bucks., England

(*Received* 23 *June* 1959)

Abstract—Acetylcholine (ACh) levels in abdominal nerve cords and thoraces of cockroaches (*Periplaneta americana*) have been measured at three stages of DDT poisoning. In the cords the ACh content, already increased at early prostration, is trebled at the late prostrate stage. In the thorax the ACh level rises even before the insect is prostrate, and has risen by 300 per cent at the late prostrate stage. Insects brought to a similar stage of prostration by physical exhaustion, or by desiccation, also contain increased amounts of ACh. The magnitude of the increase appears to be related to the degree of neuromuscular activity before prostration. It is suggested that the accumulation of ACh is the result of its release at abnormal sites in the nervous system.

INTRODUCTION

TOBIAS, KOLLROS, and SAVIT (1946) reported an increase of about 200 per cent in the acetylcholine (ACh) content of the central nervous system of the cockroach (*Periplaneta americana*) and of the fly (*Musca domestica*) at the 'prostrate, nearly motionless' stage of DDT poisoning. LEWIS (1953) concluded that the apparent increase of ACh in the fly observed by TOBIAS *et al.* (1946) was an artefact associated with their method of extraction, since he had found that the rate of synthesis in extracts of prostrate DDT-poisoned blowflies was greater than in those from the untreated flies. With the cockroach, however, the increase in ACh has been confirmed (FOWLER and LEWIS, 1958a; COLHOUN, 1959), using methods which preclude synthesis of ACh during the extraction procedure (FOWLER and LEWIS, 1958b; LEWIS and SMALLMAN, 1956).

The increase in ACh was reported by TOBIAS *et al.* (1946) to occur only in cockroaches which had reached an advanced stage of prostration. Therefore it seemed desirable to examine the effect on ACh levels of prostration produced by means other than treatment with DDT. Such a study is of particular interest in view of the recent observation that cockroaches subjected to severe mechanical or electrical stimulation reach a condition of prostration which resembles that of the DDT-poisoned insects (BEAMENT, 1958; HESLOP and RAY, 1959).

MATERIALS AND METHODS

All experiments were carried out on adult male cockroaches (*P. americana*) reared at 27°C and 40 per cent r.h., and maintained on rolled oats, toppings,

fishmeal, and yeast. Handling of the insects was facilitated by cooling them to 4°C immediately before an experiment.

Ringer's solution. NaCl 7 g, KCl 0·14 g, $CaCl_2$ 0·12 g, $NaHCO_3$ 0·2 g, glass-distilled water to 1 litre.

Mechanical prostration of cockroaches

Groups of insects were placed on a wooden turntable, being confined under perspex boxes which had hinged lids and two parallel ¼-inch strips of copper foil round the inner walls (see Fig. 1). A potential (30 V a.c.) applied across these

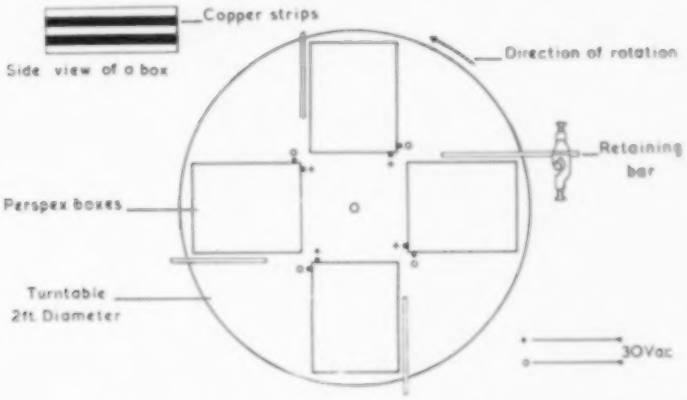


FIG. 1. Diagram showing turntable and boxes used in the mechanical prostration of cockroaches.

strips prevented the insects from staying on the walls. Thus when the turntable was slowly rotated while the boxes were held stationary the cockroaches were kept walking at the speed of the turntable and eventually became prostrate.

Removal of tissue and extraction of acetylcholine

Whole thoraces. The cockroaches were killed in liquid nitrogen, and allowed to 'warm up' in ether–solid CO_2 at about −75°C. A circular saw (3 cm diam.) driven by a 1/40 h.p. motor through a flexible drive was used to cut off the legs, head, and abdomen of each insect while it was held on a thick cork square pre-cooled with liquid nitrogen. The isolated thorax was immediately replaced in liquid nitrogen. The 'warming-up' process was found to be essential, since attempts to saw the frozen cockroaches directly after removal from liquid nitrogen resulted in their disintegration due to excessive brittleness. The thoraces were extracted by the method of FOWLER and LEWIS (1958b). Five thoraces were transferred from liquid nitrogen to 5 ml of 2 per cent TCA* in ether contained in a small lagged mortar pre-cooled with liquid nitrogen. The thoraces were ground while the temperature was kept down by periodic addition of solid carbon dioxide.

* Trichloracetic acid.

The mortar was then removed from its lagging, and the ether evaporated by means of a fine stream of air. The ether-free residue was dispersed in bicarbonate-free Ringer's solution and quantitatively transferred to a centrifuge tube, using 6 ml of the solution in all. The suspension was centrifuged (15 min at 2000 *g*) and the supernatant removed. The residue was extracted with a further 4 ml of bicarbonate-free Ringer's solution and the supernatants were combined. The extract was brought to pH 4 with 2 per cent $NaHCO_3$, and stored at $-20°C$ until required for assay.

Abdominal nerve cords. The live cockroach was fastened on its back by means of adhesive tape. A longitudinal incision was made in the abdomen on either side of the nerve cord, the strip of sternites removed, and the exposed cord gently separated from the surrounding tissue for the whole length of the abdomen. The abdominal nerve cord was raised on the prongs of an open pair of forceps, and a glass spatula, immediately pre-cooled in liquid nitrogen, was inserted under the cord freezing it instantly. The frozen cord was snapped off by lifting the spatula, and transferred to liquid nitrogen. The cords were dispersed at $-70°C$ in ethereal TCA contained in a Potter–Elvehjem homogenizer, using 2 ml of 1 per cent TCA in ether for ten cords, and extracted by the method of Fowler and Lewis (1958b).

Acetylcholine assay

The extracts were assayed on the eserinized rectus abdominis muscle of the frog, with controls to allow for the presence of sensitizing substances (Feldberg, 1945). The controls were prepared as described by Fowler and Lewis (1958b). ACh values are given in terms of *acetylcholine ion* ($C_7H_{16}O_2N^+$).

Cholinesterase estimations

Cholinesterase activity in abdominal nerve cords was measured manometrically at 25°C. Three cords were homogenized at 0°C in 1 ml of a mixture of 0·05 M sodium bicarbonate and bicarbonate-free Ringer's solution (1 : 1) in a Potter–Elvehjem homogenizer. The pestle was rinsed with 0·2 ml of the mixture, and 1 ml of the homogenate transferred to a Warburg flask containing 0·1 ml of 0·1 M acetylcholine chloride in the side arm. During the equilibration period the flasks were flushed with a mixture of 5 per cent CO_2 in nitrogen.

Chromatography of nerve cord extracts

Abdominal nerve cords, removed as described above, were homogenized at $-70°C$ in acid ethanol (500 ml ethanol : 1 ml conc. HCl), using 5 ml for sixty cords. The suspension was centrifuged for 15 min at 2000 *g*. The residue was extracted with a further 5 ml of acid ethanol and the two supernatants were combined. A flocculent white precipitate which formed after storage at $-20°C$ was centrifuged off. The volume of the extract was reduced to about 2 ml by evaporation in the cold. The walls of the tube were washed down and the volume adjusted to 3 ml with acid ethanol. Half of the extract was evaporated to dryness in the cold. The residue was taken up in 10 ml of Ringer's solution, brought to pH 7, and

assayed for ACh. The other half of the extract was chromatographed at 5°C on a paper strip (Whatman No. 1) using the propanol–formic acid–water solvent (8 : 1 : 1) of WHITTAKER and WIJESUNDERA (1952). After 24 hr the strip was dried and the chromatogram cut into ten equal parts. Each section, corresponding to 0·1 R_f, was extracted with 5 ml of Ringer's solution and the extract assayed for ACh.

RESULTS

Acetylcholine in DDT-poisoned cockroaches

The development of symptoms in cockroaches poisoned with DDT may be conveniently divided into three stages:

Tremulous. The insect twitches spontaneously and responds violently to mild stimulation, but is still able to stand and walk.

Early prostrate. The insect is on its back showing violent tremors during the first few hours after becoming prostrate. (The onset of prostration is taken as the time when an insect is first unable to right itself after being placed on its back.)

Late prostrate. The insect on its back shows only occasional feeble movements of the appendages.

VOL. 4 1960

ACh determinations have been carried out on whole thoraces from cockroaches at each of these stages, and on abdominal nerve cords of insects at the early and late prostrate stages only.

Cockroaches in groups of 30–60 were treated with 80 μg of DDT in 2 μl of acetone applied to the dorsal surface of the abdomen. The insects were confined singly under jars inverted on circles of filter paper, and kept at 25°C without food or water. Cockroaches treated with 2 μl of acetone and kept under the same conditions were used as controls. The insects were taken individually at the required stage of poisoning. When thoraces were to be extracted the whole insect was plunged into liquid nitrogen and stored until a sufficient number had been collected. If abdominal nerve cords were to be extracted these were removed immediately, as already described, and stored in liquid nitrogen. ACh content was determined on groups of five thoraces or of ten cords. The results of three such experiments are shown in Fig. 2.

As the symptoms of DDT poisoning progress the ACh content of the thorax increases, until at the late prostrate stage it may be as much as four times that of the control. Furthermore, it seems that an increase in the ACh content of the thorax can occur before the cockroaches are prostrate. Contrary to what might be expected from a consideration of the results of TOBIAS *et al.* (1946), there is an increased level of ACh in the abdominal nerve cord (60 per cent) at the early prostrate stage, 3½ hr after the onset of prostration. Similar increases were observed in thoraces from insects at this stage ½–1 hr after becoming prostrate. In the above experiments cockroaches described as early prostrate did not reach that stage until 40–55 hr after treatment with DDT. However, in experiments in which 80 μg of DDT in 2 μl of acetone was injected into the abdomen a similar stage of poisoning was reached in 4–8 hr. Each insect was plunged into liquid nitrogen ½ hr after it became

prostrate. When twenty-five insects had been collected the thoraces were extracted in groups of five. Cockroaches injected with 2 μl acetone were used as controls. These were frozen in liquid nitrogen, and the thoraces extracted in a similar

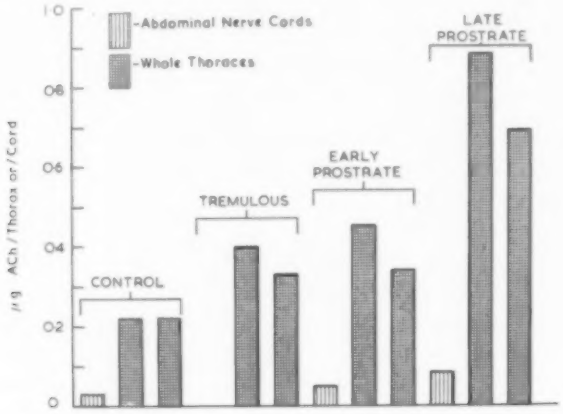


Fig. 2. The effect of DDT poisoning on the level of ACh in the abdominal nerve cord and the thorax of the cockroach.

manner. The results are shown in Table 1. Although there is a significant increase in the ACh content of the rapidly prostrated cockroaches, it is not as great as in insects which have taken a much longer time to reach a similar stage of prostration.

Table 1—Effect of rapidly induced DDT prostration on the level of ACh in cockroach thoraces

ACh (μg/thorax)		Mean increase (%)
Normal	Early prostrate	
0·26	0·36	
0·27	0·34	
0·25	0·28	
0·26	0·28	
0·20	0·28	
Mean 0·25 ±0·01 (S.E.)	0·31 ±0·02 (S.E.)	24

Significance of difference between means: $P<0{\cdot}02$ (t test).

Acetylcholine in cockroaches prostrated by mechanical means

In view of the above findings it was necessary to establish whether an increase in ACh occurs in cockroaches which become prostrate as a result of prolonged stimulation by means other than DDT.

Cockroaches were made to walk on the revolving turntable and were removed when they became prostrate (40–60 hr). Their condition deteriorated, and within

a further 48 hr it resembled that of insects at the late prostrate stage of DDT poisoning. When the insects reached this condition the ACh content of the thoraces or abdominal nerve cords was determined on groups of five thoraces or of ten cords as described in previous experiments. Equal numbers of cockroaches from the same culture were used as controls. Neither the stimulated nor the control cockroaches were given food or water during the experiment. The electrical activity of the nerve cords from mechanically prostrated cockroaches was checked with a cathode-ray oscilloscope, at the time of dissection, to ensure that only live cords were taken for extraction (from twenty-five prostrate cockroaches only three cords were found to be dead). The results of several experiments are shown in Table 2.

TABLE 2—EFFECT OF MECHANICALLY INDUCED PROSTRATION ON THE LEVEL OF ACh IN COCKROACH THORACES AND NERVE CORDS

Tissue	Expt.	ACh (μg/thorax or cord)		Mean increase (%)
		Normal	Late prostrate	
Abdominal nerve cords	1	0·032 0·036	0·066 —	94
	2	0·041 0·029	0·060 0·038	40
	3	0·033*	0·051*	55
Thoraces	4	0·14 0·17	0·32 0·29	97
	5	0·19 0·18	0·44 0·45	141

* Extracted with acidified ethanol.

The ACh content of the thoraces of the prostrate cockroaches is considerably increased, but it is not as great as that found at the late prostrate stage of DDT poisoning. The ACh level in the abdominal nerve cord is also increased, though the results are less consistent.

Acetylcholine in cockroaches prostrated by desiccation

Desiccation is a characteristic feature of cockroaches prostrated either by mechanical stimulation or by treatment with DDT. Therefore experiments were carried out to determine whether desiccation alone leads to increased levels of ACh.

Groups of fifteen cockroaches were confined without food or water over calcium chloride in a desiccator carrying inlet and outlet tubes. The air in the desiccator was replaced daily by fresh dry air introduced through a column of silica gel. The desiccated cockroaches were removed individually and frozen in liquid nitrogen when they had reached a condition resembling the late prostrate stage

of DDT poisoning (12–16 days). Cockroaches kept for a similar period without food but provided with an adequate supply of water were used as controls. ACh was determined on groups of five thoraces cut from the frozen insects. The results (Table 3) show that there is an increase in the ACh content of the thoraces

Table 3—Effect of prostration induced by desiccation on the ACh level in cockroach thoraces

Expt.	ACh (μg/thorax)		Mean increase (%)
	Normal	Late prostrate	
1	0·18 0·17	0·22 0·25	34
2	0·23 0·21	0·28 0·33	39

from the desiccated cockroaches. This increase, however, is not as striking as that found in cockroaches brought to an advanced stage of prostration by mechanical means or by DDT.

Chromatography of abdominal nerve cord extracts

Evidence for the identity of the ACh-like substance from nerve cords of mechanically prostrated cockroaches has been obtained by comparing its chromatographic behaviour with that of ACh from nerve cords of the normal insects.

Cockroaches were brought to prostration on the turntable over a period of 24–90 hr. When they reached the late prostrate stage their abdominal nerve cords were removed and stored in liquid nitrogen until sixty-one cords had been obtained. These were extracted with acidified ethanol, and half the extract was chromatographed as already described. The abdominal nerve cords from sixty normal cockroaches were extracted and half the extract was chromatographed in the same way. The distribution of the ACh-like activity along the chromatograms is shown in Fig. 3. It will be seen that the active substance from the prostrate cockroaches behaves as a single substance and has the same R_f value as that from the controls. In this experiment the level of ACh in the controls was 0·033 μg/cord and that in the prostrate insects 0·051 μg/cord as determined on the remaining half of each extract. This increase is comparable with that found in earlier experiments as shown in Table 2.

Cholinesterase activity in mechanically prostrated cockroaches

Since an increase in ACh might be attributed to cholinesterase inhibition, the cholinesterase activity in abdominal nerve cords from mechanically prostrated cockroaches was examined. Estimations were made in triplicate on cords from nine prostrate and nine control cockroaches. The mean b_{30} (μl CO_2/30 min) per

cord was 23·7 for the prostrate cockroaches and 21·3 for the controls. It is clear from these results that the increase in ACh content of mechanically prostrated cockroaches cannot be a consequence of cholinesterase inhibition.

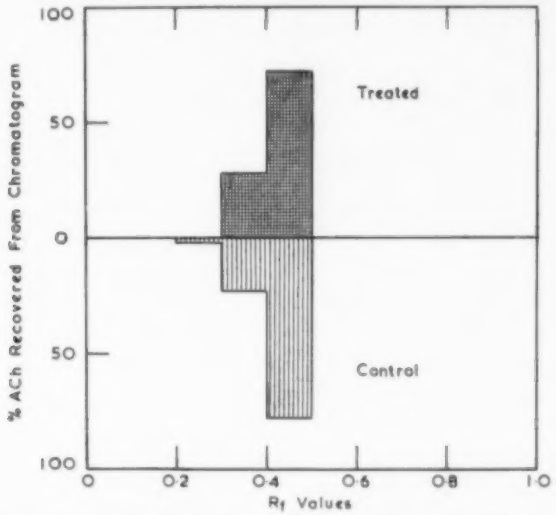


FIG. 3. The chromatographic behaviour of the ACh-like substance from nerve cords of mechanically prostrated and control cockroaches.

DISCUSSION

From the work of TOBIAS *et al.* (1946) it would appear that the increase in ACh in the thoracic nerve cord of the DDT-poisoned cockroach does not occur until the insect has reached the 'prostrate, practically immobile stage'. Similarly, COLHOUN (1959) states that a 'strikingly high increase in ACh appeared only after the roaches had been prostrate for some time'. However, our experiments both with thoraces and with abdominal nerve cords have shown that, although this condition produces the maximum rise in the level of ACh, an increase can occur in the thorax as a whole before the insect becomes prostrate. Moreover, whereas the above workers have reported that there is no increase in ACh in the thoracic nerve cord when prostration is induced rapidly, we have observed that thoraces of cockroaches prostrated rapidly by injection of DDT contained significantly more ACh than thoraces from the controls.

In making a comparison between the results obtained on the thoracic nerve cord by earlier workers and our results with the whole thorax two possible conclusions were suggested. On the one hand it could be that the previous workers lost ACh from the nerve cord during the dissection. On the other hand it may be that the increase in ACh in the whole thorax includes an increase in parts other than the central nervous system. The latter conclusion is in keeping with the results of FERNANDO (1952) showing that in cockroaches poisoned with DDT the initial accumulation of ACh is in the peripheral nervous system.

VOL. 4 1960

It is clear that in DDT-poisoned cockroaches the maximum increase in ACh in both thorax and nerve cord occurs in insects which have reached the late stage of prostration. When a similar state of prostration is induced by mechanical means there is again an increase in the level of ACh both in the abdominal nerve cord and in the thorax. A much smaller increase was observed in thoraces from insects prostrated by depriving them of food and water. Unlike the mechanically prostrated cockroaches these had not passed through a period of abnormal neuromuscular activity before prostration. It may be concluded from these results that part of the increase in ACh at the late prostrate stage of DDT poisoning is concomitant with the physiological state of the insect, and is neither a direct consequence of DDT action nor of excessive neuromuscular activity. Nevertheless, there does appear to be some connexion between the magnitude of the increase at the late prostrate stage and the degree of neuromuscular activity before prostration.

The increase in ACh in the DDT-poisoned insect occurs although cholinesterase activity, as measured *in vitro*, is unimpaired (Tobias *et al.*, 1946). Similarly we have found that the cholinesterase of nerve cords from mechanically prostrated insects is not inhibited. These observations raise the question of the identity of the substance accumulating in the prostrate insects which has been assayed as ACh on the frog muscle. Tobias *et al.* (1946) showed that the substance from the DDT-poisoned insect is destroyed by alkali and by cholinesterase, and is inactive on the curarized frog muscle. We have observed that the substance which accumulates in the mechanically prostrated insects also resembles ACh in that it is destroyed by alkali, and in its action on the frog muscle. Furthermore, it was not separated by chromatography from the ACh-like substance in nerve cords from normal cockroaches. Spencer and O'Brien (1957), quoting Chang and Kearns (1955), conclude that ACh is the only pharmacologically active compound in nerve cords from normal cockroaches. Thus, although the evidence is not conclusive, it would seem likely that the pharmacological activity in the mechanically prostrated cockroaches can be attributed to a single substance and that this is ACh.

The question arises how such abnormal amounts of ACh can accumulate in tissues in which cholinesterase activity is unimpaired. Obviously the excess ACh is not available to cholinesterase and in this respect resembles ACh in normal tissue, but it does not follow that it exists in the same form. In fact, the results of Tobias *et al.* (1946) and of Colhoun (1959) on the extraction of ACh with eserinized Ringer's solution show that ACh in DDT-poisoned insects is more easily extractable than that in normal insects. Tobias *et al.* (1946) suggested that the increase in ACh in DDT-poisoned insects is the result of its liberation from a bound form, probably by the action of some metabolic intermediary, and it seems to us that this offers the most likely explanation of the results reported here. However, since the excess ACh is not immediately destroyed by cholinesterase, it is necessary to postulate that it is released at abnormal sites in the nervous system.

Acknowledgements—We wish to thank Mr. J. W. Ray for his assistance in checking the electrical activity of the abdominal nerve cords.

REFERENCES

BEAMENT J. W. L. (1958) A paralysing agent in the blood of cockroaches. *J. Ins. Physiol.* **2**, 199–214.

CHANG S. C. and KEARNS C. W. (1955) *3rd Meeting Ent. Soc. America, Cincinnati, Ohio.* Not seen by authors.

COLHOUN E. H. (1959) Acetylcholine in *Periplaneta americana* L.—III. Acetylcholine in roaches treated with tetraethyl pyrophosphate and 2 : 2-bis (*p*-chlorophenyl)-1, 1, 1-trichloroethane. *Canad. J. Biochem. Physiol.* **37**, 259–272.

FELDBERG W. (1945) Synthesis of acetylcholine by tissue of the central nervous system. *J. Physiol.* **103**, 367–402.

FERNANDO E. H. (1952) *The acetylcholine-like substance in insect nervous tissue and its role in the toxicity of chlorinated hydrocarbon insecticides.* Ph.D. thesis, University of Illinois, Urbana.

FOWLER K. S. and LEWIS S. E. (1958a) In *Pest Infestation Research* 1957, p. 37. H.M.S.O., London.

FOWLER K. S. and LEWIS S. E. (1958b) The extraction of acetylcholine from frozen insect tissue. *J. Physiol.* **142**, 165–172.

HESLOP J. P. and RAY J. W. (1959) The reaction of the cockroach, *Periplaneta americana*, to bodily stress and DDT. *J. Ins. Physiol.* **3**, 395–401.

LEWIS S. E. (1953) Acetylcholine in blowflies. *Nature, Lond.* **127**, 1004.

LEWIS S. E. and SMALLMAN B. N. (1956) The estimation of acetylcholine in insects. *J. Physiol.* **134**, 241–256.

SPENCER E. Y. and O'BRIEN R. D. (1957) Chemistry and mode of action of organophosphorus insecticides. *Annu. Rev. Ent.* **2**, 261–278.

TOBIAS J. M., KOLLROS J. J., and SAVIT J. (1946) Acetylcholine and related substances in the cockroach, fly and crayfish and the effect of DDT. *J. cell. comp. Physiol.* **28**, 159–185.

WHITTAKER V. P. and WIJESUNDERA S. (1952) The separation of esters of choline by filter-paper chromatography. *Biochem. J.* **51**, 348–351.

VOL. 4 1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 138 *to* 145. *Pergamon Press Ltd., London. Printed in Great Britain*

ACTION POTENTIALS IN THE ANTENNAE OF THE BLOWFLY (*CALLIPHORA ERYTHROCEPHALA*) DURING MECHANICAL STIMULATION*

DIETRICH BURKHARDT

Zoologisches Institut, Universität München

(*Received* 1 *July* 1959)

Abstract—The excitation patterns in Johnston's Organ during mechanical stimulation were studied by means of microelectrodes in *Calliphora erythrocephala*. An electromechanical transducer was used to deliver stimuli with a rectangular time course, whereby the funiculus of the antenna was rotated about its long axis. By variation of stimulus intensity, polarity, duration, and frequency, the build-up of excitation patterns may be investigated while the antenna is subjected to a variety of stimulus patterns.

The recorded action potentials lead one to the conclusion that the majority of nervous elements are responding only to movements, each phase of movement causing a single spike. Some of the elements observed respond to torsional movements independent of direction; others are sensitive only to movements in one of the two possible directions. During stimuli of short duration, strong interactions between the action potentials released at the beginning and end of stimulation may be observed. These interactions are responsible for the observation that slight changes in stimulus pattern cause major changes in excitation pattern during high frequency repeated stimulation (up to 500 c/s).

Because of the numerical preponderance of phasic receptors in the funiculus–pedicellus joint, the antenna of the blowfly is a sense organ specialized for sensitive time resolution of air flow stimuli. The antenna therefore exhibits some properties of a hearing organ: it responds to airborne sound of frequencies up to 500 c/s. Especially in the case of single stimulation with short rectangular impulses (click), the potentials are very similar to the nervous component of the cochlear potential of birds released by the same sort of stimuli.

In a previous investigation (Burkhardt and Schneider, 1957) it was shown by behavioural experiments and electrophysiological measurements that the antennae of the blowfly are sensitive, extraordinarily fast-reacting current sense organs. Antennal excitation is involved in regulation of the animal's speed in free flight. A constant stream of air forces the arista to act like a lever arm which turns the funiculus outwards around its long axis. A series of action potentials is thereby released in Johnston's Organ. In the cited investigations, action potentials were also detected when the animal was subjected to airborne sound at frequencies below 500 c/s (cf. Westecker, 1957). However, all attempts to establish behavioural reactions to airborne sound failed. In many animals, there is a close relationship between current sense and hearing, and there is reason to believe that in the course of phylogeny many hearing organs have developed from organs originally used

* These investigations were supported by a grant from the Deutschen Forschungsgemeinschaft.

for current detection in vertebrates and insects (SCHWARTZKOPFF, 1959). The properties of the *Calliphora* antennae lead one to suppose that they represent a transitional stage between organs for current sense and hearing. A thorough electrophysiological investigation seems to be particularly interesting here, especially if the build-up of excitation by static and dynamic deflexions (imitating stimuli of current flow and airborne sound) can be followed with microelectrodes.

The experimental animals were taken from cultures of the Zoological Institute in München. Males and females were used equally often, as no differences could be observed in their responses. Steel needle or tungsten microelectrodes (BURKHARDT, 1954; HUBEL, 1957) were used to lead off potentials from the pedicellus, in the medial part of Johnston's Organ; the action potential was amplified, observed, and recorded synchronously with the stimulus on a double-beam oscilloscope (Tectronix Type 502).

For stimulation, the arista or the funiculus was moved by the thinly drawn out, bent tip of a glass rod. The glass rod was glued to a small loudspeaker which was fed from a Grass Stimulator S4. The stimulator delivers rectangular electrical impulses of any desired strength, polarity, and duration, singly or repeated at selected intervals.

As in natural stimuli, the funiculus was rotated about its long axis by the artificial stimulus. It was rotated abruptly, maintained its position for the length of the stimulus, and then returned abruptly to its original position. Stimuli of current flow can thus be imitated in a simple way by single, longer deflexions. By increasing stimulus frequency and shortening stimulus duration the build-up of excitation during rhythmic stimulation (in imitation of natural sound stimulation) can be followed up to high frequencies. The loudspeaker employed had a resonance frequency of 350 c/s, therefore a low-amplitude damped oscillation of this frequency was superimposed on the beginning and end of each rectangular stimulation.

VOL. 4 1960

In Fig. 1 a typical action potential is shown which arises from a single rotation of the antenna. After a short latency (0·5–2 msec at 20°C) the preparation responds to stimulus onset with a large, di- (or tri-) phasic action potential. Latency and amplitude of this 'on-wave' are dependent on stimulus strength (angle of rotation) (see Fig. 2); the amplitude may be greater than 5 mV. A series of smaller, usually mono- or diphasic action potentials follows the on-wave; their amplitude decreases during the course of stimulation. A comparison with the movements of the stimulating needle shows that this series of action potentials is released by the superimposed oscillations. Towards the end of such a series, single action potentials may drop out of this rigidly stimulus-synchronized succession. During low-frequency repetitive stimulation, the amplitude of the large on-wave often fluctuates stepwise, in a random manner, within a considerable fraction of its maximum range (Fig. 3). Both facts may be explained as follows: the individual action potentials, under microelectrode leadoffs, are composed of a small number of rigidly synchronized discharges of single nerve elements. With increasing stimulus intensity, the number of synchronously discharging elements increases (and thereby also the amplitude of the registered potentials). Towards the end of the damped oscillations, only a few elements are still subjected to a stimulation strength above threshold. If threshold happens to vary a little, or stimulus strength changes slightly, part of these potentials may drop out. The action potentials of the

single elements obey the all-or-none law. Until now, however, it has not been possible to decide if these single discharges originate from the soma or the axons of the sensory nerve cells.

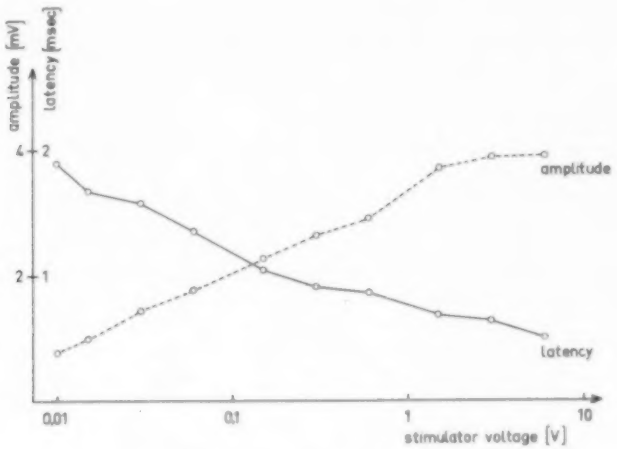


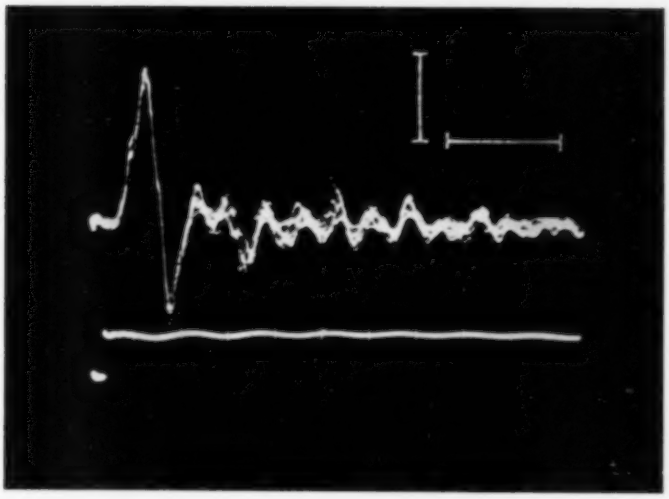
FIG. 2. Dependence of amplitude and latency of the on-wave upon stimulus intensity. As a measure of stimulus intensity, the voltage on the stimulator is plotted on the abscissa (log scale); the deflection is proportional to the voltage. Latent period is measured from stimulus onset to beginning of on-wave; amplitude is the difference in height between the highest positive and negative deflections (cf. Fig. 1).

During continued deflexion of the antenna no change in activity, compared with that before the stimulus, can usually be observed. Since, in addition, the action potential and the course of movement of the stimulating needle are strictly synchronous, it can be concluded that the elements described here respond only to movement, but not to constant deflexion (phasic receptors). Each phase of movement releases a single action potential with the rapid stimuli employed.

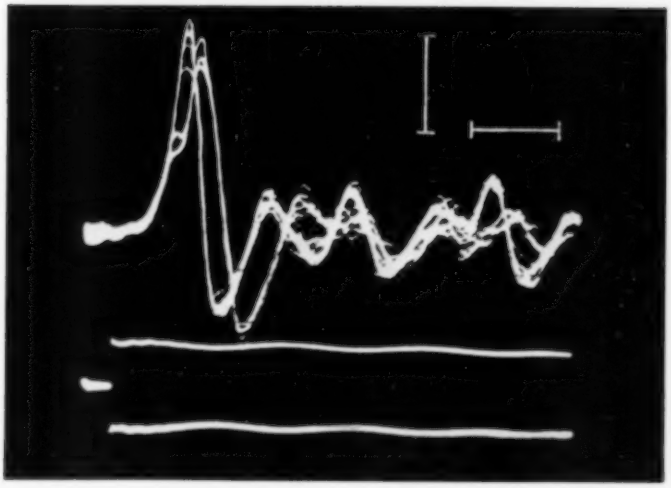
At the end of the stimulus, an action potential is obtained which is very similar to that obtained when the stimulus begins. If the traces of on- and off-waves are superimposed on the oscilloscope, one can see that in certain phases the action potentials are congruent, whereas in others they differ from one another (cf. Fig. 3). If the direction of rotation of the antenna is reversed, the action potential at the onset of the stimulus now looks like the potential at the end of the stimulus for the original direction of antennal rotation, and *vice versa*: at the end of the stimulus it appears similar to the previous trace for the onset of the stimulus. In general, the amplitudes of the on-waves for a given rotation direction are smaller, and their latency is somewhat larger, than for the opposite direction of rotation.

The partial similarities of the action potentials for various directions of rotation may be hypothetically explained as follows: some of the elements respond only to turning in one direction, whereas others respond to every movement in any direction.

VOL.
4
1960



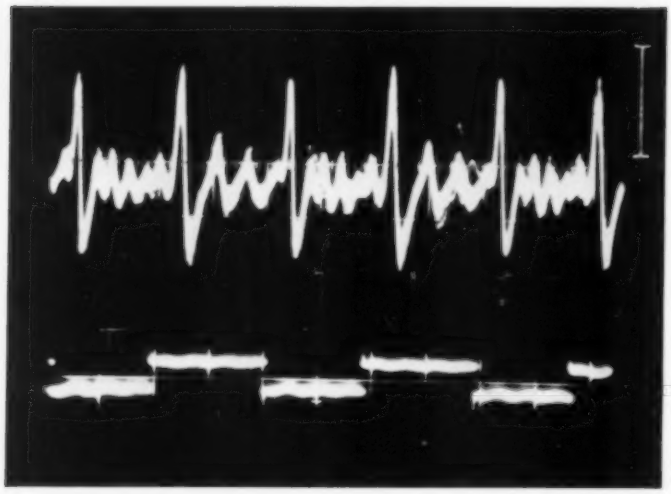
(1)



(3)

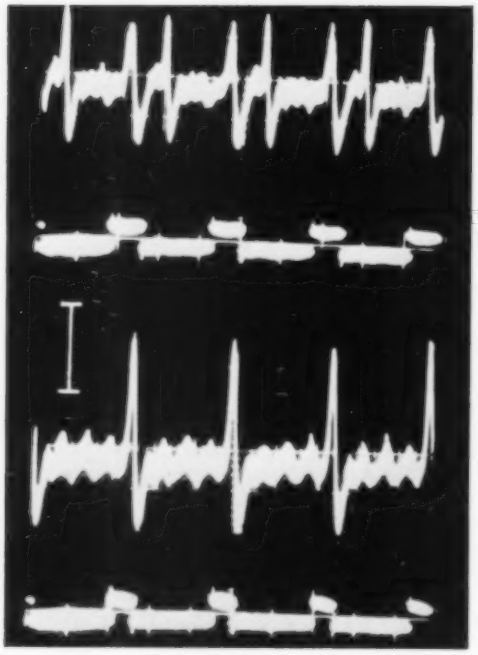
FIG. 1. Phasic action potential in response to abrupt rotation of the antennae. Calibration: horizontal 5 msec, vertical 1 mV. Lower trace: time course of the stimulus. Upper trace: left, the on-wave (diphasic in this case), followed by smaller action potentials which have doubled the frequency of the damped oscillations superimposed on the stimulus (cf. lower trace). For the photograph action potentials of several stimuli were registered superimposed on one another.

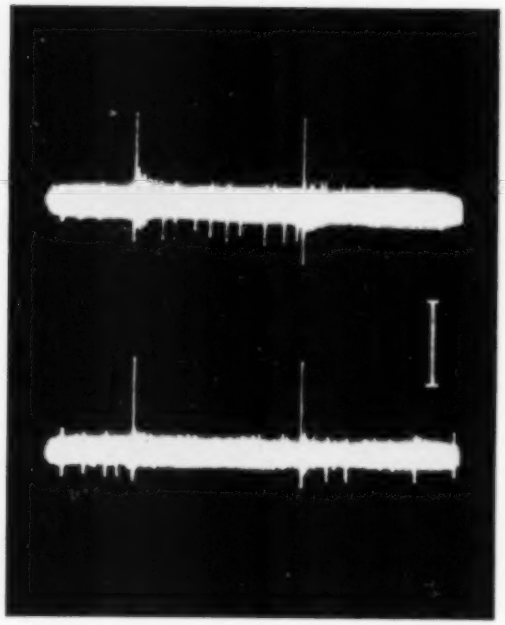
FIG. 3. Upper trace: superimposed on-waves from stimuli of opposite polarities. Lower trace: time course of the corresponding stimuli. Calibration: horizontal 2 msec, vertical 1 mV. The action potentials of the two oppositely directed stimuli are congruent in certain phases. Similar pictures are observed if on- and off-waves are superimposed. Falling phase of the on-wave retraced for purposes of reproduction.



(4)

(6)





(7)

FIG. 4. Action current picture with rhythmic stimulation (upper trace). Stimulus: lower trace. Stimulus frequency 50 c/s, duration 9 msec. Calibration: 2 mV.

FIG. 6. Action potentials during constant stimulus frequency and slightly varied stimulus duration. Upper trace: action potential. Lower trace: stimulus. Frequency for both records 80 c/s. Stimulus duration for upper record ca. 4 msec, lower record ca. 3 msec. Calibration: 2 mV. In the lower record, the off-waves are suppressed and the on-waves fully developed.

FIG. 7. Tonic discharge of position receptors. The two records show the action potentials during stimuli of opposite polarities. Stimulus duration always 0·8 sec. Calibration: 2 mV. At the beginning and the end of stimulus, the very high phasic action potentials are visible. Tonic elements are visible between them in the upper record; before and after them in the lower record. Peaks retraced for better reproduction.

This is also supported by the interaction between on- and off-waves during short stimulation (to be described later).

This response pattern of the nervous elements can be explained by the geometrical arrangement: in general a mechanical stress in a given direction (for example stretching) serves as a stimulus. The receptors, however, have different orientations with respect to the axis of rotation. Thus some will be stimulated only by a certain direction of rotation, some by both possible directions of rotation.

In order to investigate the build-up of excitation by rhythmic stimulation, the stimulus frequency is increased during constant short stimulus duration. Up to intervals of 20–30 msec, the action potentials for single stimuli are hardly changed. The investigated movement receptors do not seem to show adaptation. With further shortening of the intervals between stimuli, the on-wave of each stimulus approaches more and more closely the off-wave of the preceding stimulus. When successive intervals are exactly twice as long as stimulus duration, one observes regular rows of alternating on- and off-waves. These action potentials then look exactly like those released by airborne sound; the action potential frequency is equal to the doubled stimulus frequency (WESTECKER, 1957). The potentials described in the investigations of BURKHARDT and SCHNEIDER as arising from airborne sound, thus seem to arise from the rigidly synchronized discharges at the beginning and ending of each torsional vibration stimulus. It is safe to assume, however, that these potentials are not an effect comparable with the microphonic component of the cochlear potential of birds and mammals. These potentials of the insect antenna are much more similar to the nervous component of the cochlear potential, the discharge of the first acoustic neurones. Especially in single stimulation with short rectangular impulses ('click') of alternating polarities, the potentials show a striking similarity to those registered when the bird cochlea is subjected to click stimuli of alternating polarities (SCHWARTZKOPFF, 1958). In birds also the superimposed potentials of opposite polarities are only partially congruent; the amplitude is greater and the latency shorter for one of the two polarities. PUMPHREY (1940) has already pointed out the similarities between the action potentials in certain insect hearing organs and those of the higher vertebrates.

If the stimulus duration is shortened, while the frequency is held constant and not too high, interactions between the on- and off-waves can be observed. With increasing short stimulus duration, the off-wave approaches more closely the series of small action potentials released by the damped oscillations of the stimulator, and finally the on-wave. It does not simply superimpose on these potentials; it is suppressed at certain stimulus durations, but develops fully at others. The latency between stimulus end and off-wave also changes during this shortening as follows: with small off-wave amplitudes a long latency is registered; with larger amplitude latency is shorter (Fig. 5). The height of the discharge—and thus probably the number of synchronously discharging elements—is thus determined for the off-wave by the preceding discharges. These shifts in latency are such that the off-wave tends to occupy the position of one of the previous discharges. For example when stimulus duration is shortened, the latency is at first longer, so that it remains

VOL. 4 1960

approximately at the same position where a discharge occurred with longer stimulus duration. When the stimulus duration is further shortened, the latency decreases abruptly, so that now the off-wave occupies the previous position of an earlier-occurring discharge. One must therefore assume strong interactions between the nervous elements participating in the on- and off-effects.

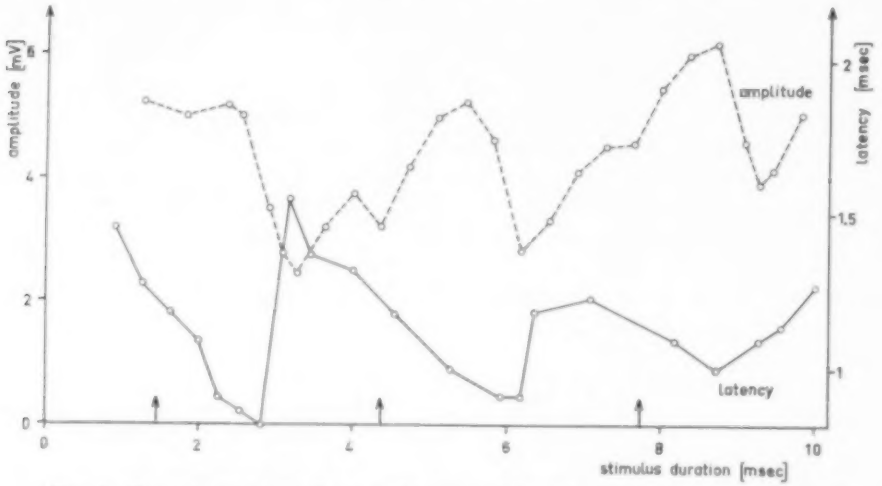


FIG. 5. Dependence of amplitude and latency of the off-wave upon stimulus duration (with short stimuli). The first arrow on the abscissa indicates the time of occurrence of the peak of the on-wave; the following arrows those of the later smaller action potentials. The momentary location of the off-effect on the abscissa can be calculated as follows: the latent period (the ordinate value) must be added to stimulus duration. When this is done, the abscissa must be viewed as the time co-ordinate. Because of the shifts in latency, the off-waves tend to occupy the position of preceding discharges; their amplitude thereby changes in the opposite direction to latency.

Two possibilities might be considered here: (1) the elements participating in the on- and off-effects inhibit each other reciprocally; or (2) the same elements participate in the on- and off-effects; therefore these elements are refractory after the on-wave for a certain period and cannot contribute to the off-wave. By means of periodic stimulation with small variations in stimulus duration or frequency, one can obtain either an action potential synchronous with the stimulus (by suppressing the off-wave) or one with a frequency twice that of stimulation (when the off-wave is fully developed), because of the described interaction (Fig. 6).

By means of a certain shortening of the stimulus, the off-wave is suppressed as it approaches the on-wave. In a similar way, by appropriate prolongation of the stimulus, the on-wave of the succeeding stimulus can be suppressed as the preceding off-wave approaches more closely. By small opposite variations in stimulus duration, the on- and off-effects can thus be alternately suppressed; by this means the phase of the action potential can be shifted almost 180° with respect to the stimulus while maintaining the same frequency.

Minute changes in the stimulus pattern thus cause extreme alterations in the spatio–temporal excitation pattern in Johnston's Organ. Such nervous effects can only be analysed by isolated investigations of single or few active elements with microelectrodes, and by a careful study of the excitation pattern. A summary starting from the total organ and an evaluation of the effect from a formal physical viewpoint ('Klirrfactor' of TISCHNER, 1953 and KEPPLER, 1958) is not suitable for the biological function of such a complexly-built organ.

During rectangular stimuli of long duration, it was sometimes possible to record spike discharges of single nervous elements throughout the duration of the stimulus, in addition to the described on- and off-waves. When the direction of rotation was reversed, this discharge was visible before and after the stimulus, but during the stimulus it was obliterated (Fig. 7). In addition to the phasic receptors responding only to movements therefore, the antennae of *Calliphora* also possess tonic receptors which react to the deflexion itself (BURKHARDT and SCHNEIDER, 1957; in *Locusta migratoria* UCHIYAMA and KATSUKI, 1956). However, these receptors seem to be very rare. On the basis of present experiments it cannot yet be decided whether we are dealing with elements of Johnston's Organ or with other position receptors. STADTMÜLLER (1955) describes innervated hair tufts between funiculus and pedicellus, which might conceivably participate in the indication of position.

VOL. 4 1960

By stronger single deflexions of the antenna, one frequently observes a dense, irregular series of relatively large action potentials about 200 msec after the stimulus onset. Observations with the binocular microscope show that the antennae are thereby actively moved. Probably these action potentials are muscular action currents. Apparently the position of the antennae is actively changed by the animal under strong stimulation (cf. HERAN, 1957).

In a previous work (BURKHARDT and SCHNEIDER, 1957) it could be shown that when the antennae are subjected to a steady air current, irregular spike-like potentials arise. The antennae are made to tremble by the air-flow (by air eddies ?). This result could be confirmed by leading off with microelectrodes. The oscillograms give the impression that during the stimulus by air current, the antenna reacts with an irregular pattern of on- and off-waves (phasic action potentials), similar to the waves which respond regularly to airborne sound. The majority of receptors in the antenna are phasic receptors which respond only to movement. The question arises therefore, whether it is possible that these phasic receptors are also capable of registering mean deflexion of the antennae. It is thought that this could be made possible by the following mechanism: by continuous vibration of the antennae the phasic receptors are continuously re-stimulated. With increasing angle of rotation the number of elements excited also increases. In addition the peak of excitation in the spatial pattern of the sensory cells will shift with various angles of rotation. Thus, through the spatio–temporal pattern of excitation of Johnston's Organ, the CNS can receive exact information about the strength and time course of the antennal deflexion.

A very exact resolution of the time course of air flow stimuli is ensured by the preponderance of phasic receptors in the antennal joint between funiculus and pedicellus. Thus it is not surprising that this kind of current sense organ possesses a great many of the properties of a hearing organ, as shown by the investigations of the build-up of excitation with rhythmic stimulation and comparisons with the bird ear.

ZUSAMMENFASSUNG

Die Erregungsmuster im Johnston'schen Organ von *Calliphora erythrocephala* werden mit Mikroelektroden bei mechanischer Reizung der Antenne untersucht: Ein elektromechanischer Reizgeber dreht den Funiculus wie bei natürlichen Reizen um seine Längsachse; der Zeitverlauf des Reizes ist rechteckförmig. Durch Veränderung von Intensität, Polarität, Dauer und Folgefrequenz der Reize kann der Aufbau der Erregungsmuster bei verschiedenen Reizmustern verfolgt werden.

Aus den abgeleiteten Aktionspotentialen muss geschlossen werden, dass die überwiegende Mehrzahl der nervösen Elemente nur auf Bewegungen anspricht und zwar auf jede Bewegungsphase mit einem einzigen Spike: phasische Receptoren. Ein Teil der Elemente scheint auf jede Bewegung unabhängig von der Drehrichtung anzusprechen, ein Teil nur auf Bewegungen mit einen jeweils bestimmten Drehsinn. Bei kurzer Reizdauer können starke Wechselwirkungen zwischen den durch Reizbeginn und Reizende ausgelösten Aktionspotentialen beobachtet werden. Diese Wechselwirkungen bedingen, dass bei periodischer hochfrequenter Reizung (bis 500 Hz) geringfügige Änderungen des Verhältnisses zwischen Reizdauer und Pausendauer einschneidende Veränderungen des Erregungmusters zur Folge haben.

Durch das Überwiegen phasischer Receptoren im Funiculus-Pedicellus-Gelenk ist die Fliegenantenne ein Sinnesorgan, welches auf eine besonders genaue zeitliche Auflösung von Strömungsreizen spezialisiert ist. Diese Spezialisierung bedingt, dass die Antenne bereits Eigenschaften eines Gehörorgans besitzt: Sie spricht auf Luftschall bis zu Frequenzen von 500 Hz an, und die Erregungsvorgänge bei einmaligen kurzen Rechteckreizen (Klicks) zeigen auffällige Parallelitäten zu den nervösen Komponenten des Cochleapotentials der Vögel unter gleichen Reizbedingungen.

REFERENCES

BURKHARDT D. (1954) Rhythmische Erregungen in den optischen Zentren von *Calliphora erythrocephala*. *Z. vergl. Physiol.* **36**, 595–630.

BURKHARDT D. und SCHNEIDER G. (1957) Die Antennen von *Calliphora* als Anzeiger der Fluggeschwindigkeit. *Z. Naturf.* **12b**, 139–143.

HERAN H. (1957) Die Bienenantenne als Messorgan der Fluggeschwindigkeit. *Naturwissenschaften* **44**, 475.

HUBEL D. H. (1957) A tungsten microelectrode for recording from single units. *Science* **125**, 549–550.

KEPPLER E. (1958) Über das Richtungshören von Stechmücken. *Z. Naturf.* **13b**, 286–284.

PUMPHREY R. J. (1940) Hearing in insects. *Biol. Rev.* **15**, 107–132.

SCHWARTZKOPFF J. (1958) Über den Einfluss der Bewegungsrichtung der Basilarmembran auf die Ausbildung der Cochleapotentiale von *Strix varia* (Barton) und *Melopsittacus undulatus* (Shaw). *Z. vergl. Physiol.* **41**, 35–48.

SCHWARTZKOPFF J. (1959) Der akustische Reiz und die Gehörserrregung. *Verh. dtsch. zool. Ges, Münster* 1959, in press.

STADTMÜLLER R. (1955) *Untersuchungen zur Anatomie und Histologie der Antennen von* Calliphora erythrocephala. Zulassungsarbeit für das Staatsexamen, Würzburg.

TISCHNER H. (1953) Über den Gehörsinn der Stechmücken. *Acustica* **3**, 335–343.

UCHIYAMA H. and KATSUKI Y. (1956) Recording of action potentials from the antenneal nerve of locusts by means of microelectrodes. *Physiol. comp.* **4**, 154–163.

WESTECKER M. (1957) *Elektrophysiologische Untersuchungen der Antennenreaktion von* Calliphora erythrocephala *bei Luftschall.* Inauguraldissertation, Würzburg.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 146 *to* 153. *Pergamon Press Ltd., London. Printed in Great Britain*

PROTEOLYTIC ENZYMES FROM THE MIDGUT OF THE HOUSEFLY*

NARAYAN G. PATEL and A. GLENN RICHARDS

Department of Entomology and Economic Zoology, University of Minnesota, St. Paul, Minnesota

(*Received* 20 *July* 1959)

Abstract—Three electrophoretically separate bands hydrolysing gelatin-agar substrate were found in extracts from the midgut of adult houseflies. One of these seems to be an endoenzyme from the tissues of the gut, probably from epithelial cells. The other two enzymes are from the midgut contents. These enzymes also have characteristic differences in heat denaturation and substrate-pH sensitivity.

INTRODUCTION

LITTLE information is available regarding the digestive mechanism and specific enzymes involved in the utilization of different kinds of food in insect diets, and only a few attempts have been made to study the nature and mechanism of protein digestion in particular. CHAMPLAIN and FISK (1956) have reported some qualitative identifications of digestive enzymes from midguts of the adult stable fly, *Stomoxys calcitrans* L. Recently PATTERSON and FISK (1958) have reported trypsin-like activity from the same insect. LIN and RICHARDS (1955) presented reaction velocity curves, which were linear within experimental error, for proteases from the housefly and American cockroach. The above authors all used homogenates of midguts for their studies, but their data were consistent with the assumption that they were measuring the activity of a single enzyme. However, HUNT and MOORE (1958) have shown that there is a complex protease system in bacteria. They obtained a single well-defined peak in a plot of hydrolysis versus pH, but found three separate peaks from electrophoretic separation of the same proteolytic enzyme complex obtained from a gram negative rod isolated from the bovine rumen. Three electrophoretically separable trypsin-like substances have recently been reported for midgut homogenates from the stable fly by PATTERSON and FISK (1958). The present qualitative study reveals a similar complexity in the proteolytic enzymes from midguts of the adult housefly, *Musca domestica* L.

MATERIALS AND METHODS

(1) *Preparation of enzyme extract*

Larvae of a DDT-susceptible strain of housefly were reared on wheat bran–alfalfa mixture at 37°C and 60 per cent humidity. After the flies had emerged,

* Paper No. 4187, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1, Minnesota.

they were fed sugar and water for 5 days. On the sixth day, at least 12 hr before dissection, milk was fed overnight. The flies were chilled for about 5 min before dissection. Twenty midguts were removed in the cold and transferred immediately to chilled 1 ml sodium phosphate buffer at pH 4·5, and another twenty midguts were prepared at pH 8·0. Three such vials were held at about 5°C; one vial was removed after 6 hr, the second after 12 hr, and the third after 48 hr. It was expected that during this period of cold storage the digestive juice would have diffused into the buffer. Centrifugally separated fractions of supernatant and sediment were kept at 5°C and used later as the sources of enzymes.

(2) *Electrophoresis*

LKB type 327 paper electrophoresis equipment was used with a Heathkit power supply. Four 40×425 mm strips of filter paper were used, marked at 126 mm from the anode end for the point of application. The strips were soaked in sodium phosphate buffer and laid on the plastic platform for 5 min to equilibrate with the buffer in the electrode vessels. The pH of the buffer system was varied, and accordingly will be stated in the text.

VOL. 4 1960

At the end of 5 min, excess of the buffer was blotted up with a clean filter paper strip, and about 50 μl of the crude enzyme was applied as a streak at the marked point. A current of 320 V and about 25–30 mA was applied; this gave about 0·15–0·2 mA/mm of cross-section of the paper. The electrophoresis was run in the cold for 4 hr.

(3) *Detection of the separated components*

(a) *Gelatin hydrolysis.* Gelatin (1 per cent) and agar (1·5 per cent) was dissolved in boiling water or buffer, and poured in a shallow pyrex tray 15×25 cm, and allowed to solidify in the cold for 10 min. Wet paper strips removed from the electrophoresis apparatus were placed on the gelatin-agar slab and incubated for 6 hr at 37°C. The unhydrolysed gelatin was precipitated with acidified $HgCl_2$ (15 g $HgCl_2$ dissolved in 100 ml H_2O and 20 ml conc. HCl); non-precipitated bands, which represent hydrolysis of the gelatin, were traced on their respective filter paper strips.

(b) *A modified technique of pH gradient for gelatin-agar.* A modification of the above technique was used for determining inhibition of proteolytic activity at various pHs of the substrate. To get a gradient of pH values, gelatin-agar in high pH buffer was poured on a solidified slant with low pH (Fig. 1). It was necessary to pour the second buffer on the top of the first which had just solidified in order to achieve a satisfactory pH gradient. For qualitative purposes, the pH gradient was checked by change of colour of a strip of hydronium (pH) paper, but for more precise determination small blocks of 1×1 in. were removed from the gelatin-agar slab, placed in test tubes in 2 ml of distilled water and the pH of this water measured with a Beckman model H2 pH meter after 24 hr. Gradients of the required acidic or alkaline range could be obtained by manipulation of pH of the buffer and slope of

the slants (Fig. 1, B and C). Instead of four strips, one larger sheet of paper (LKB, Munktell No. 205) was used for electrophoretic migration of enzymes for the pH gradient studies.

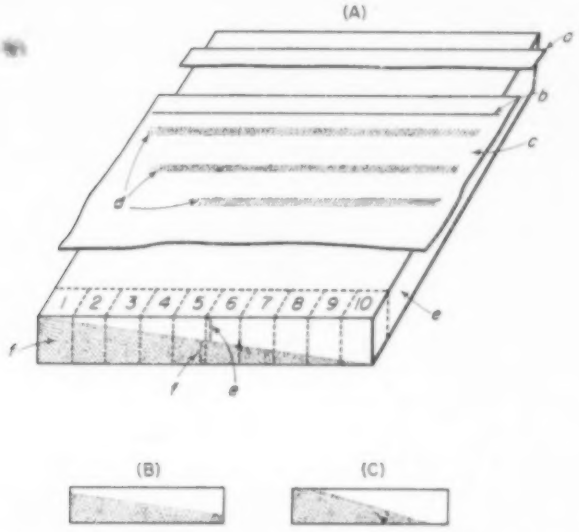


FIG. 1. A diagrammatic presentation of a method for obtaining a pH gradient on a gelatin-agar slab.

A. Details of pH gradient with normal slants (with pH range from 5·0 to 7·9)
   a. Hydronium (pH) paperstrip
   b. Original line of application
   c. Electrophoretic sheet
   d. Position of enzymatically active bands
   e. Alkaline buffer
   f. Acidific buffer
   1, 2, 3, . . . indicate the blocks removed to measure pH with pH meter.

B. Change of the slope to give a narrower range of pH (6·6–7·1) with increased distance between two successive pH values.

C. Steeper slope to give broader range (4·6–8·3) with decreased distance in between two successive pH values.

(c) *Fluorescent pattern under ultra-violet (UV) light.* Fluorescence was observed to find out the relative position of the enzymatically active bands prior to the hydrolysis tests. Strips were dried at 37°C for an hour, exposed to UV light, and the fluorescing bands marked. Fluorescent compounds often serve as indicators for the migration of non-fluorescent ones in homogenates or crude extracts.

(d) *Heat denaturation.* Approximately 50 μl aliquots of the extract were put in glass capillary tubes which were then heat-sealed quickly at one end. Capillaries were tied to the mercury bulb of a thermometer which was submerged in a water bath. Temperature was raised in such a way that a 2·0°C increase was attained in 5 min time. After reaching the required temperature, the heating was discontinued

and temperature was maintained steady (±0·5°C) for 10 min and then the capillaries were cooled in cold water. The contents were then run in the electrophoresis apparatus and subsequently tested for enzymatic activity.

(e) *Preparation of known proteolytic enzymes.* Sodium phosphate buffer of pH 5·5, 0·1 M was used to prepare 10·0 per cent pepsin (DIFCO), 10·0 per cent papain (DIFCO), 10·0 per cent rennin (DIFCO), and 0·3 per cent cathepsin-C (SIGMA).

Some trypsin (1·0 per cent) (DIFCO) was prepared with sodium phosphate buffer of pH 8·0 and 0·1 M strength. The pH optima of these enzymes do not correspond to the pH of the buffer used for their preparation, but it was desired to have the pH during electrophoresis the same as that of the extracts prepared from the midguts.

RESULTS

1. *Electrophoretic mobility.* The gelatin hydrolysis showed three clearcut bands migrating towards the cathode from 48 hr extracts at pH 8·0. These three bands will be referred to as the 'stationary', 'slow', and 'fast' bands in reference to their mobility from the original application point towards the cathode (Fig. 2A—a, b, and c respectively). The 12 hr extract showed the same pattern (Fig. 2B) but the stationary band was weak. The 6 hr extract showed only the slow and fast bands (Fig. 2C). The same pattern was observed when 6 hr extracts in pH 5·5 buffer were run with pH 8·0 buffer in the electrophoretic cells; however, when this alkaline buffer was replaced with pH 5·5 in the cells the fast band disappeared. This gave an indication that the fast band is comparatively more susceptible to acidic conditions.

The disappearance and decrease in the intensity of the stationary band with decreased length of extraction time suggested that this enzyme is not released with the slow and fast bands, i.e. it is, probably, not present in the juice from the beginning, but has come later from some other source. One possibility is that it might be an endoenzyme released later from midgut epithelial cells. This was checked by removing juice directly from the midgut with a very fine micro capillary. The contents were run on electrophoresis. There was one very faint band coinciding with the slow band but the activity was so low that it could not be accepted as conclusive evidence. To overcome this technical difficulty of obtaining a sufficient amount of juice directly from midguts, midguts were allowed to soak in pH 8·0 buffer for 12 and 48 hr in cold, the guts were centrifuged out from the supernatant and transferred to fresh buffer. This second extract was used after 6 hr. Only the stationary band gave positive hydrolysis (Fig. 2D). This is what one would expect if the slow and fast bands come from the juices in the lumen of the gut but the stationary band from lysis of the midgut cells.

2. *Fluorescent pattern.* Three fluorescent bands could be seen under UV light; none of these bands coincided with the proteolytic activity of the enzymes (Fig. 2E). Of the three bands, the central one fluoresced yellow, the others light blue. These fluorescent bands were used to determine the relative position of enzyme bands.

VOL. 4 1960

3. *Hydrolysis of substrate on pH gradient.* The modified technique of pH gradient was used to determine the loss of hydrolysing power of the enzyme. It was found that the stationary band did not lose its potency even down to pH 4·6, whereas the slow band disappeared at about pH 5·3, and the fast band was active

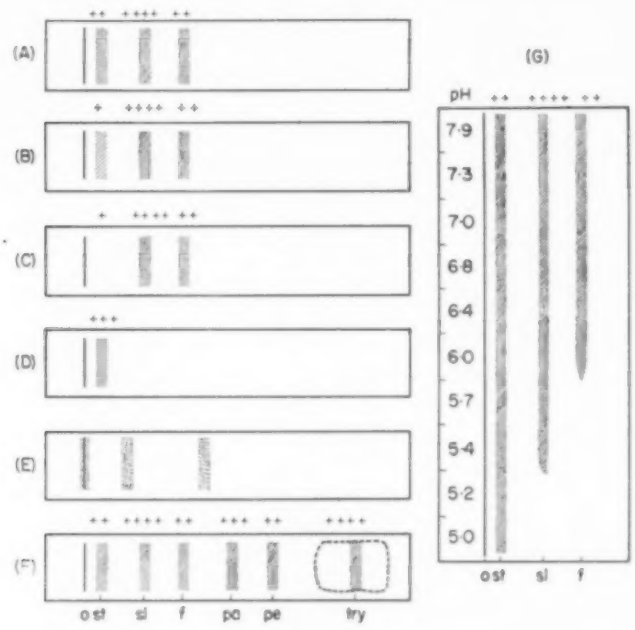


FIG. 2.

A, B, and C. Electrophoretic pattern of 48 hr, 12 hr, and 6 hr extracts respectively.
D. Electrophoretic pattern of second extract from the tissue of midgut from which the digestive juice was removed by initial extraction.
E. Fluorescent pattern of 48 hr extract.
F. Relative electrophoretic migration of extract from midgut, papain, pepsin, and trypsin.
G. Hydrolysis pattern on pH gradient of the three enzymatic bands of midgut extract.

\+ = Positive hydrolysis; number of signs indicates the relative amount of hydrolysis
± = little or doubtful hydrolysis
— = no hydrolysis
o = original line where the extract was applied
st = stationary band
sl = slow band
f = fast band
pa = papain
pe = pepsin (pig)
try = trypsin (beef).

only down to about 5·9 in the acidic range (Fig. 2G). This behaviour of pH sensitivity was confirmed with regular gelatin-agar substrate of pH 4·6, 5·3, and 5·9 in separate trays.

4. *Heat denaturation.* The results are shown in the table.

TABLE 1—HEAT DENATURATION OF PROTEOLYTIC ENZYMES FROM MIDGUT OF ADULT HOUSEFLIES

10 min incubation (°C)	Relative activity		
	Stationary band	Slow band	Fast band
unheated control	+ + + +	+ + + +	+ + + +
65	+ +	+	+ + +
70	+	—	+ +
75	±	—	+
80	—	—	—

\+ Positive hydrolysis, number of crosses indicates relative intensity.
± Doubtful hydrolysis.
— No hydrolysis.

For the heat denaturation studies only 48 hr supernatant in pH 8·0 was used.

5. *Histology.* A cursory histological examination* did not give any detailed information; however, it was fairly clear that midguts which were fixed immediately after the dissection had normal orientation of the epithelial cells on the circular muscles of the midgut wall. Midguts which were kept in the cold overnight (12 hr) in pH 8·0 showed a delamination of the epithelial cells from the circular muscles. Some of these cells were found floating freely in the midgut cavity, others were ruptured and their nuclei dispersed. Microscopically similar cells were found in the sediments which gave the stationary band. Few or no free cells were in sediments after only 6 hr in the cold buffer solution.

6. *Mobility comparison with known enzymes.* Cathepsin and rennin did not hydrolyse gelatin under the experimental conditions used. Papain, pepsin, and trypsin showed bands of hydrolysed gelatin but their relative migration did not show any correlation with the bands from the midgut of houseflies (Fig. 2F). They migrated respectively 30, 50, and 90 per cent more than the fast band from fly extracts.

DISCUSSION

An enzyme source which gives a clear-cut single peak in pH and hydrolysis curves often does not give a true picture of the complexity involved, particularly when the source is a 'homogenate' or 'crude extract' of some kind. HUNT and MOORE (1958) reported such complexity of the proteolytic system from gram negative rods of bovine rumen. Similarly, a pH optimum curve with one well-defined peak for proteolytic enzyme activity related to *Bacillus larvae* infecting honey bee larvae was found by GOCHNAUER (1956) when he used crude extracts of dead larvae; but later PATEL and GOCHNAUER (1958) showed that the crude extract had two well-defined proteolytic enzymes, which can be separated by electrophoretic and chromatographic techniques. Such observations indicate that it would be fruitful

* See 'Acknowledgement', p. 152.

VOL. 4 1960

to determine the complexity of any enzyme system before a detailed investigation, more so when the source used is not an isolated fraction.

It was suspected that there might be a similar situation in the juice of housefly midguts. Hence different test criteria were used for qualitative determination of proteolytic enzymes in the present work. Our attempt has apparently shown that from midgut juice we have two proteases which can hydrolyse gelatin; a third is present in extracts but seems to come from the lysis of midgut cells. Presumably, all three would be present in homogenates. And, indeed, three components were recently separated electrophoretically from homogenates of stable fly midguts by PATTERSON and FISK (1958). It is quite possible that these enzymes may have different substrate preferences. It is also possible that other proteases are present but were not detected because of preference for other substrates or because for some reason they fail to hydrolyse gelatin under the conditions used.

In the present work no attempt has been made to discern the origin of the enzymes, except for that represented by the stationary band on electrophoresis. The release of this enzyme coincides with the separation of epithelial cells of the midgut from the circular muscles and rupturing of some of the cells. The origin of the other two has not been traced. It is possible that one of them might be related to microflora of the midgut, as abundant growth of bacteria was observed in some midgut extracts.

The pH gradient technique used in the present work did not permit determination of the pH optimum or the shape of the pH activity curve but it did show that the three enzymes differ from one another although all have maximum activity at near neutrality and above. This technique has the definite advantage that one can work with micro-quantities and crude mixtures which have more than one protease.

SUMMARY

(1) Paper electrophoretic migration of crude extracts from midguts of adult houseflies shows three bands of proteolytic activity on gelatin-agar substrate. These seem similar to the three trypsin-like components recently separated from homogenates of stable fly midguts by PATTERSON and FISK (1958). All three components differ in electrophoretic mobility from mammalian pepsin and trypsin.

(2) Of the three proteolytic enzymes, the one which is nearly stationary seems to be an endoenzyme from the epithelial cells.

(3) These enzymes differ in their ability to hydrolyse substrate at different pH. The one which migrates the least from the original spot hydrolyses the substrate even down to pH 4·6, whereas the slow-moving one is not active below pH 5·3, and the fast-moving one is active only to pH 5·9.

(4) The stationary band loses its activity when it is heated for 10 min at about 75°C, whereas the slow and fast are denatured at 70 and 60°C respectively.

Acknowledgement—Thanks are due to Dr. RUDOLPH L. PIPA for preparation of the histological slides.

REFERENCES

CHAMPLAIN R. A. and FISK F. W. (1956) The digestive enzymes of the stable fly, *Stomoxys calcitrans* L. *Ohio J. Sci.* **56**, 52–62.

GOCHNAUER T. A. (1956) Unpublished data.

HUNT W. G. and MOORE R. O. (1958) The proteolytic system of a gram negative rod isolated from the bovine rumen. *Appl. Microbiol.* **6**, 36–39.

LIN S. and RICHARDS A. G. (1956) A comparison of two digestive enzymes in the housefly and American cockroach. *Ann. ent. Soc. Amer.* **49**, 239–241.

PATEL N. G. and GOCHNAUER T. A. (1958) Separation of two proteolytic enzymes from honey bee larvae infected by *Bacillus larvae*. *Bact. Proc.* p. 118.

PATTERSON R. A. and FISK F. W. (1958) A study of the trypsin-like protease of the adult stable fly, *Stomoxys calcitrans* L. *Ohio J. Sci.* **58**, 299–310.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 154 *to* 175. *Pergamon Press Ltd., London. Printed in Great Britain*

THE ROLE OF PHOTOPERIOD AND TEMPERATURE IN THE DETERMINATION OF PARTHOGENETIC AND SEXUAL FORMS IN THE APHID *MEGOURA VICIAE* BUCKTON—II. THE OPERATION OF THE 'INTERVAL TIMER' IN YOUNG CLONES

A. D. LEES

Agricultural Research Council Unit of Insect Physiology, Cambridge

(*Received* 21 *August* 1959)

Abstract—The ability of young clones of *Megoura* to form sexuales (males and oviparae) under short day conditions is regulated by a timing mechanism which at first prevents the appearance of these morphs. The operation of this 'interval timer' is unaffected by the number of generations through which the lineage has passed. It is, however, influenced by temperature, though not by the type of parents (apterous or alate virginoparae) used in the intermediate generations. Under permanent long day conditions the 'interval timer' operates at the appropriate moment, but the potentiality for forming oviparae remains latent until evoked by short days.

The timing mechanism is probably located in the cytoplasm. Since the number of individual- and cell-generations does not influence its rate of operation, it is suggested that the elements involved may be capable of self-duplication while undergoing continuous transformation. The latter change must culminate in the restitution of the maternal control system which permits the egg or embryo to develop along alternative pathways in response to the appropriate environmental signals.

INTRODUCTION

In his classical demonstration of the photoperiodic control of sexual reproduction in aphids Marcovitch (1924) observed that the spring generations did not immediately yield sexuales when exposed to short day lengths. For example, under the conditions prevailing in Tennessee *Aphis forbesi* hatched from overwintering eggs in February, yet could not be induced to form oviparae until May. Species with host alternation (*Aphis rumicis*, *A.* (*Sappaphis*) *sorbi*) which received short days from April onwards on the secondary host, failed to produce sexuales until June. Although strikingly premature in relation to the appearance of these forms in nature, several parthenogenetic generations had therefore intervened before the treatment became effective. Marcovitch concluded that the 'length of day is apparently not operative when certain stages are predetermined, and follow in quick succession'.

Wilson (1938) obtained similar results with *Aphis chloris*. When the fundatrices and their descendants were placed on a short day régime the appearance of males and oviparae was delayed until the seventh generation. Thereafter the proportion of oviparous offspring increased up to the tenth generation when it reached 100 per cent. In these experiments each fresh generation was started with an early-born

offspring of the previous one. But WILSON estimated that the generation time would have been approximately doubled if a later-born offspring had been selected. He further pointed out that as in nature the sexual forms tend to be produced over a relatively limited period, individual oviparae which develop synchronously may nevertheless be preceded by a highly variable number of parthenogenetic generations. He therefore postulated the existence of a 'time factor', transcending the generations, whose influence waned as the post-fundatrix interval lengthened. With the earlier spring generations usually developing in short days and moderately low temperatures, the adaptive function was clearly to inhibit the appearance of sexuales at the very beginning of the annual cycle.

This concept of a 'time factor' or 'facteur fondatrice' was supported by BONNEMAISON (1949, 1951) who set up two lineages of *Brevicoryne brassicae*, one emanating from a larva born early in each generation and the other from one born late. These 'accelerated' and 'retarded' lines were exposed from the time of hatching (at the end of January) to a 12 hr photoperiod, then to a natural (long) photoperiod from 9 March to mid-June, and subsequently to an artificial photoperiod of 8 or 13 hr (both short). In response to this treatment the first males were born about 28 April in both lineages when the accelerated line had passed through seven generations and the retarded line only five. Information regarding the production of oviparae was less complete since the short day régime was discontinued before they appeared. Nevertheless, both the accelerated and the retarded lines proved to be capable of producing oviparae when they were again exposed to an artificial short photoperiod of 8 hr in July.

The vetch aphid *Megoura viciae* has already been used for studying the role of photoperiod and temperature in the production of parthenogenetic and sexual forms, the parent aphids in this case being apterous virginoparae from a clone that had remained in the parthogenetic condition for some hundreds of generations (LEES, 1959). The present results are concerned with the influence of these same agencies on fundatrices and on the succeeding generations of virginoparae. The establishment of accelerated and retarded lineages—or 'first-born' and 'last-born' lineages as they are here called—has yielded some further information on the operation of the 'time factor'. For reasons which are considered later (p. 172), the latter is referred to in this paper as the 'interval timer'.

ORIGIN AND MAINTENANCE OF THE LINEAGES

Sexuales were obtained by exposing apterous parents from the long-established parthogenetic clone (normally kept at 15°C in a 16 hr photoperiod) to a short day of 12 hr. The fertilized oviparae were then scattered over a dense mat of *Lathyrus pratenis* growing in an unheated garden frame. Diapause eggs were laid during September on the soil near the base of the plants and these began to hatch about 1 April. All the fundatrices used in the following experiments were collected as second or third instar larvae from the young growing points of the vetch during the period 6–12 April. They were immediately transferred to the appropriate experimental conditions.

The aphids were reared in cabinets with controlled temperature and illumination, as described elsewhere (Lees, 1959). When adult, each fundatrix (= generation 1) was allotted a separate broad bean (*Vicia faba*) plant and was subsequently transferred twice to fresh plants during the period of reproduction. Three successive batches of offspring were thus obtained. 'First-born' and 'last-born' lineages were next initiated by selecting the largest apterous virginopara from the first batch and the smallest from the last batch. These generation 2 individuals were treated differently, only the most advanced offspring of the first batch being required as the parent of generation 3 in the 'first-born' lineage; similarly, only the most retarded larva in the third batch was saved for continuing the 'last-born' lineage. This procedure was then repeated at each generation so that the principal lines of descent from each fundatrix never exceeded two in number. Since size was taken as a criterion of age, the terms 'first-born' and 'last-born' are of course approximations. It was also necessary, for ease of handling, to transfer the aphids simultaneously. The selection of the larva for continuing the 'last-born' lineage was then dictated by the productivity of the most infertile parent. For this reason the generation time in the 'last-born' lineage tends to be rather less than the time span obtainable from a normally fertile individual.

Although only one 'first-born' and one 'last-born' individual was used for perpetuating the lineages, the operation of the 'interval timer' could be followed more closely by rearing the progeny of several mothers of similar age. Naturally, the capacities of virginoparae as male- or ovipara-producers can only be tested at intervals of a generation. But since it was found that mothers of closely similar age often become sexuales-producers at slightly different rates, this provided a more accurate index of the state of the clone. In practice, 4–6 additional virginoparae were reared in each generation, both in the 'first-born' and 'last-born' lineages. These individuals were chosen for their similar size (and therefore, presumably, age) to the virginopara selected for continuing the lineage. After examination, their families were discarded.

Since the fundatrices were only collected in the third instar, the onset of parturition was used as the point of reference in the life cycle. It has been shown previously that in females from a long-established clone the production of sexuales is controlled maternally, the form of the offspring being decided before birth (Lees, 1959). It follows that the time occupied by the development of the offspring should be omitted in calculating the 'time interval from the fundatrix'. In tabulating the observations the time lapse between the onset of parturition in the fundatrix and the same event in the *parent* has therefore been taken as standard. On the other hand, the generation number, as given in the Tables, applies to the progeny. The following example from Table 1 will clarify this matter. Fundatrix 'A' (generation 1) produced 25 second-generation offspring. The first of these 25 daughters began to reproduce after 15 days and ultimately gave birth to the 23 individuals that comprised the third generation.

Apterous virginoparae were used as parents except in the instances mentioned below. Alatae occurred in many of the virginoparous generations and were

particularly prevalent in some clones. Since the origin of these winged forms depends on factors entirely different from those under consideration here, it has often been convenient to group alatae and apterous individuals as 'virginoparae'.

VOL.
4
1960

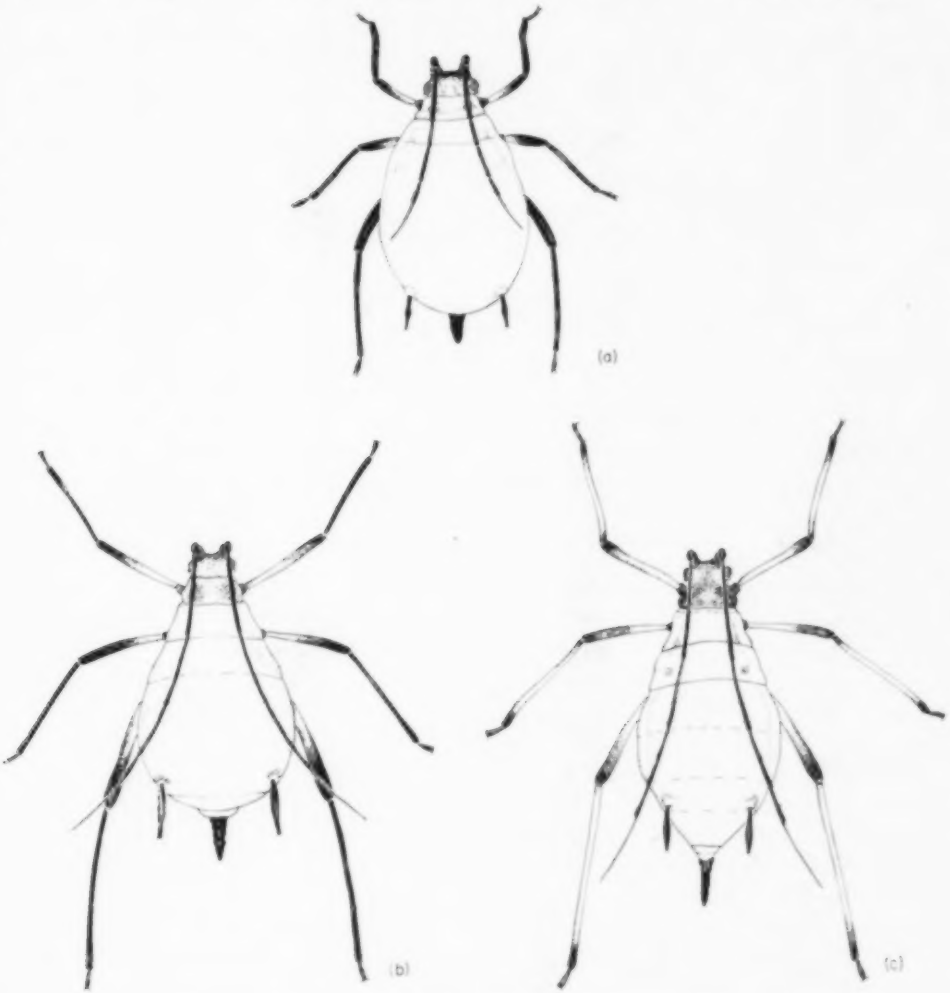


FIG. 1. *Megoura viciae* Buckton. (a) fundatrix; (b) apterous virginopara from the generation following the fundatrix; (c) apterous virginopara from a long-established (10 year) clone.

In classifying the morphs no distinction has been made between the virginoparae of the first and subsequent generations after the fundatrix, despite the fact that slight morphological differences exist. The general appearance of these forms will be conveyed by Fig. 1 which shows the fundatrix (a), a virginopara of generation 2

(i.e. a daughter of the fundatrix) (b), and an apterous virginopara from the long-established stock clone (c).

FECUNDITY OF THE LINEAGES

The marked clonal differences in fecundity which were encountered have an indirect bearing on form determination and must therefore be briefly mentioned.

Unlike certain aphid species, the fundatrices of *Megoura* are not appreciably more fecund than the apterous virginoparae of succeeding generations. In the case of 26 fundatrices reared at 15°C the mean number of offspring per parent was 70. This compares with a value of 97, previously given for the apterous virginoparae of the long-established parthenogenetic stock (LEES, 1959). However, the first figure has little meaning since at least 5 individuals (38 per cent) were obviously sub-fertile. It was noticeable that this characteristic was usually transmitted to all the descendants within the clone. Fundatrix 'A' (Table 1), for example, produced 25 offspring; and the 32 families reared in subsequent generations of this clone averaged only 35 individuals. On the other hand, fundatrix 'B', which gave birth to 111 daughters (Table 1), yielded 92 offspring per parent in a total of 27 families. This clone is probably representative of a normally fertile parthenogenetic lineage of *Megoura*. It seems probable that the high proportion of sub-fertile fundatrices may have been due to the presence of a deleterious recessive gene in the original stock. Such a gene would remain in the heterozygous condition in a clone reproducing by diploid parthenogenesis. However, as the fertilized eggs are the products of brother–sister matings one-quarter of the fundatrices should be homozygous for this gene.

A decline in fecundity also took place, even in normally productive clones, after the aphids had been reared for two or three generations at 11°C. Such changes often occur in temperatures that are below (or above) the optimum range (LEES, 1959). Although the primary effect is doubtless on the parent, the reduction in fecundity is first manifested in the progeny. The cause is unknown.

Previous work with the long-established parthenogenetic clone has shown that at 15°C the first 20 or so offspring are almost invariably female. The relatively few males, varying from 0 to 22 per parent, are born principally during the middle reproductive life of the parent. It follows that in sub-fertile clones the parent can invariably be classified as an ovipara- or a virginopara-producer. But it may not be possible to detect whether the female is a potential male-producer since the progeny sequence may stop short of the point where male determination is most probable. For example, in clone 'A' only one male was recorded in 32 families.

RESULTS

The production of sexuales in short day conditions at 15°C

'First-born' and 'last-born' lineages derived from six fundatrices were exposed continuously to a 12-hr photoperiod at 15°C until males and oviparae appeared. The composition of the families from which the parents of subsequent generations were selected is listed in Table 1. Several general features may be noted.

TABLE 1—PROGENY RECORDS FOR THE SIX CLONES WHICH WERE EXPOSED CONTINUOUSLY TO A 12-HR DAY AT 15°C. APTEROUS VIRGINOPARAE FROM THESE FAMILIES WERE CHOSEN TO PROVIDE THE LINES OF DESCENT IN THE 'FIRST-BORN' AND 'LAST-BORN' LINEAGES

(V., virginoparae; Ov., oviparae; ♂, males)

Fundatrix (gen. 1) and progeny (gen. 2)	Days	15	30	35	55	66	76	96	99
	Gen. no. 'First-born'	3		4	5		6		7
	Gen. no. 'Last-born'		3			4		5	
'A' 25 V.	'First-born'	23 V.		83 V.	46 V.		44 V.		29 Ov.
	'Last-born'		90 V.			57 V.		35 Ov.	
'B' 111 V.	'First-born'	126 V.		128 V.	84 V. 2 ♂		71 Ov. 9 ♂		
	'Last-born'		126 V.			70 V. 9 ♂		75 Ov. 17 ♂	
'C' 98 V.	'First-born'	101 V.		120 V.	95 V. 3 ♂		23 Ov.		
	'Last-born'		100 V.			79 V. 10 ♂		6 V. 98 Ov. 11 ♂	
'D' 107 V.	'First-born'	101 V.		114 V.	118 V.		91 V. 2 ♂		71 Ov. 5 ♂
	'Last-born'		136 V.			98 V.		45 Ov. 3 ♂	
'E' 86 V.	'First-born'	90 V.		104 V. 4 ♂	36 V. 36 Ov. 4 ♂		73 Ov. 17 ♂		
	'Last-born'		118 V.			43 Ov.			
'F' 79 V.	'First-born'	128 V.		89 V.	54 V. 10 Ov.		87 Ov. 2 ♂		
	'Last-born'		119 V. 3 ♂			55 V. 9 ♂		90 Ov. 5 ♂	

VOL. 4 1960

Although this environment should be highly conducive to the production of sexuales (LEES, 1959), no males or oviparae were in fact present in the generations immediately following the fundatrix: these consisted of virginoparae only. In the 'first-born' lineages the oviparae appeared initially in generation 5 (clones 'E' and 'F'), 6 (clones 'B' and 'C'), or 7 (clones 'A' and 'D'); whereas in the 'last-born' lineage the first oviparae were recorded in generations 4 (clone 'E') or 5 (the remaining clones). Sometimes, as in clone 'A', oviparae were formed first in the 'last-born'

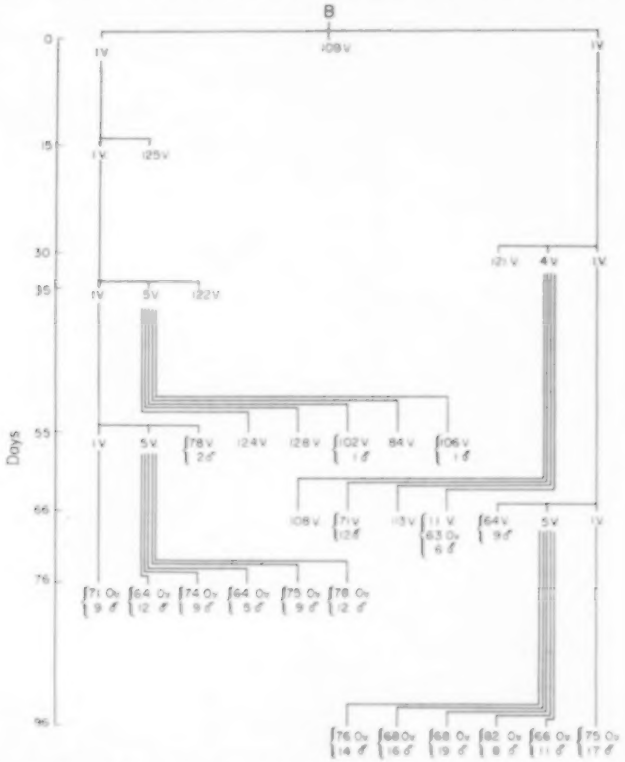


FIG. 2. The genealogy of clone 'B'. The time axis is vertical, as in a family tree. The time scale refers only to the virginoparae selected as parents. The latter are indicated separately, older parents to the left, younger parents to the right. It follows that the 'first-born' and 'last-born' lineages also lie to the left and right respectively. The aphids were exposed throughout to a 12-hr photoperiod at 15°C. *V.*, virginoparae; *Ov.*, oviparae; ♂, males.

lineage, despite the disparity in generations. Males often appeared rather earlier than oviparae, occurring in generations 4, 5, or 6 of the 'first-born' lineage and in generations 3, 4, or 5 of the 'last-born' lineage.

These results accord with BONNEMAISON's (1951) conclusions and suggest strongly that the capacity of the parent to respond to short days is independent of generation number. At the same time it appears probable from Table 1 that the

ability to form sexuales is a function of the age of the clone. This is most apparent when both 'first-' and 'last-born' lineages are available for testing at the time the transition is going forward.

More information can be derived from these observations if the responses of the additional virginoparae tested in each generation are included. These supplementary individuals (close sisters of the virginoparae selected for continuing the lineage—see p. 156), were first reared at 30 days in the third generation of the

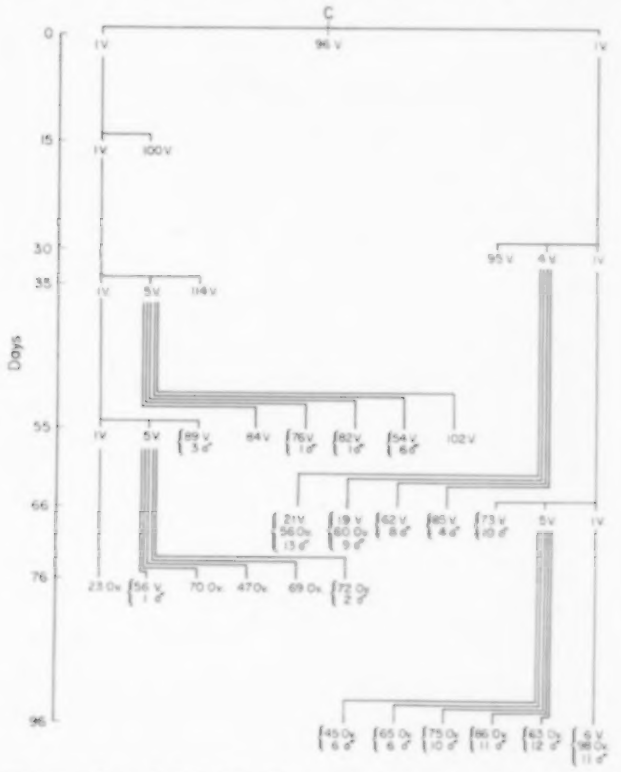


FIG. 3. The genealogy of clone 'C'. The conventions used are the same as in Fig. 2.

'last-born' lineage when it was suspected that the production of sexuales was about to begin. Thereafter supplementary individuals were reared in each generation. The full genealogies of clones 'B', 'C', and 'E' are given in Figs. 2, 3, and 4.

The 'interval timer'. An examination of these expanded lineages suggests that the time factor is more constant than Table 1 would suggest. Clone 'B' may be cited in illustration. It will be seen that in the fifth 'first-born' generation all six of the parents were virginopara-producers after a time interval of 55 days. At 66 days, in generation 4 of the 'last-born' lineage, four out of five parents still produced virginoparae exclusively. Nevertheless, as one yielded both virginoparae

and oviparae, the transition was beginning to take place—a conclusion that would not emerge from Table 1 since the parent selected for continuing the lineage happened to be one of the four virginopara-producers. Ten days later, at 76 days in the 'first-born' lineage, the change had been completed, all the parents producing

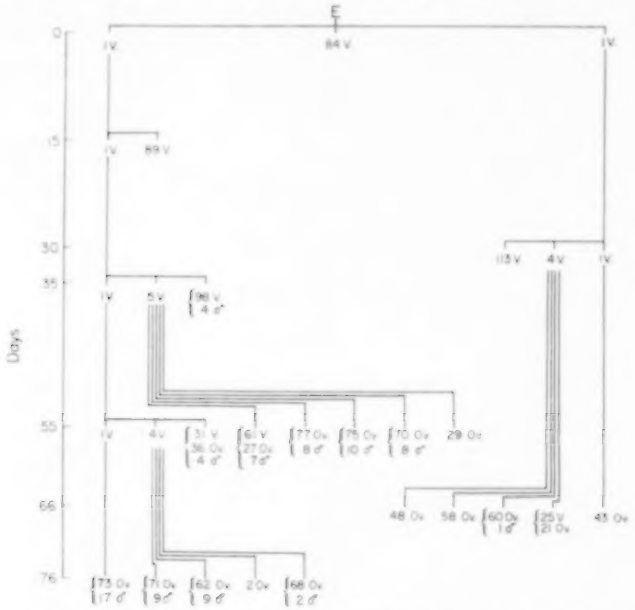


FIG. 4. The genealogy of clone 'E'. The conventions used are the same as in Fig. 2.

oviparae only. In the 'last-born' lineage there was no new generation ready for reproduction and testing until the ninety-sixth day. All these parents proved to be exclusively ovipara-producers, the transition having meanwhile been completed in this lineage also.

The reappearance of the capacity to form males also takes no account of generation. The age of the clone was again the governing factor, the time interval being somewhat less than in the case of oviparae. If the generations happened to be appropriately spaced relative to the transition, males sometimes appeared first in the retarded lineage (e.g. clone 'F', Table 1). But the state of the clone with respect to male determination can again be better appreciated by considering the performance of several parents of identical age. In clone 'B', for example, it is seen that after a time interval of 55 days three out of six parents in generation 5 of the 'first-born' lineage were male-producers. Eleven days later in the fourth 'last-born' generation the proportion was three out of five; and ten days later again in the sixth 'first-born' generation all six parents yielded males (Fig. 2).

This type of response can be traced in other clones such as 'C' and 'E', although poor fecundity sometimes obscures their potential as male-producers. For instance, two 'first-born' parents in generation 6 of clone 'C' produced families of 23 and

47 oviparae respectively. Since male-production had already begun in the previous generation some 21 days before, the implication is that males would have featured in these families if the sequence of offspring had been longer (see p. 158).

INTRA- AND INTER-CLONAL VARIATIONS IN THE 'INTERVAL TIMER'

The results discussed in the previous section suggest that the timing mechanism operates with impressive regularity in different lineages within a clone. This point is illustrated in Fig. 5 which traces the proportion of parents that were capable of producing either oviparae exclusively or mixed families of virginoparae and oviparae at the different intervals of time. Despite the limitations of periodic sampling, there seems no doubt that the overt transition begins almost simultaneously in the parallel lineages. And although the evidence is more fragmentary, the same type of regularities appear to characterize the production of males.

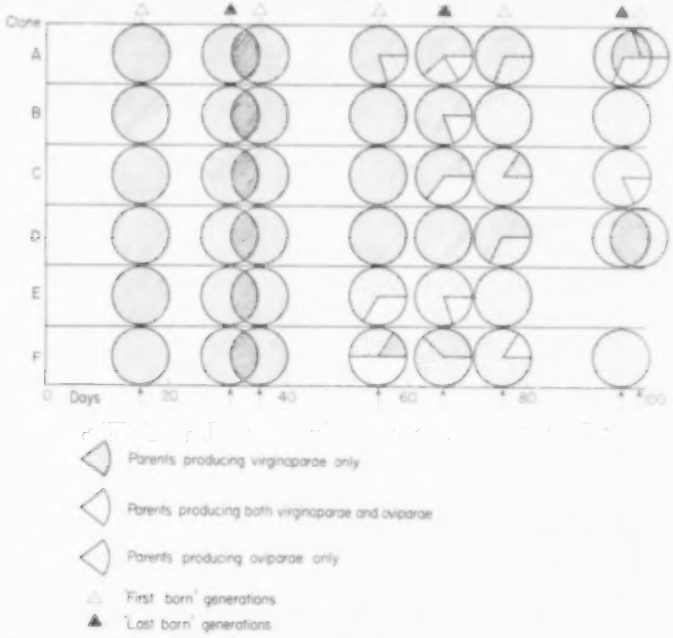


FIG. 5. The response of six young clones to constant short day conditions at 15°C. The figure illustrates how the ability of parent aphids to produce oviparae is acquired with the passage of time. The diagrams indicate the proportions of the three classes of parent represented in each generation.

It is also clear from Fig. 5 that the transition from incipient to fully developed gamic reproduction may require several generations and a time interval of over 50 days. This is seen in clone 'A', for example. However, the rate of transition seems to be similar in both lineages and is evidently a property of the clone. This is referred to again below.

VOL. 4 1960

Differences between clones are much more striking. Some clones acquired the ability to form sexuales relatively early. In 'E', for example, the first males appeared in the fourth 'first-born' generation after 35 days; and the fifth generation at 55 days included the first oviparae, four out of six fourth-generation parents producing oviparae only and two producing mixed families of virginoparae and oviparae (Fig. 4). In clone 'F' the sexuales also developed early. In clone 'B', however, the first males had only just begun to appear after 55 days; and only a small proportion of the parents in the fourth 'last-born' generation had begun to form oviparae after 66 days (Fig. 2). The transition was still further delayed in clone 'D', the first males being born after 66 days and the first oviparae after 76 days in the sixth 'first-born' generation.

As mentioned above, the rate of transition, once initiated, exhibits notable clonal differences. In clone 'B' the function was acquired rapidly. Fig. 2 shows that this clone was just beginning to be male-producing after 55 days and that this faculty was apparently fully developed by 76 days. Similarly, the transition from incipient to fully developed ovipara-production was virtually confined to the period from 66 to 76 days. The transition was comparatively slow in clones 'A' and 'C'. In the first of these, one-fifth of the parents from the fifth 'first-born' generation proved to be ovipara-producers after 55 days; yet even after 99 days the transition was still incomplete in one-third of the parents. These continued to give birth to virginoparae (Fig. 5).

A study of inter-clonal variation might be expected to throw some light on whether the processes of male- and ovipara-production are causally related. The fact that males usually precede the oviparae in time suggests at first sight that the processes are independent, and that they are controlled by two 'interval timers' with different constants. Yet there is apparently some degree of association since in 'fast' clones, such as 'E', both males and oviparae appear relatively early, whereas in 'slow' clones, such as 'D', both morphs appear late.

THE 'INTERVAL TIMER' IN RELATION TO THE INDIVIDUAL CYCLE OF DEVELOPMENT

At what ontogenetic stage is the capacity to produce oviparae restored? In the case of a long-established parthenogenetic stock of *Megoura* it has been established that at birth none of the embryos present in the first instar larva (there are usually two in each of the eighteen ovarioles) are finally committed to become virginoparae or oviparae. The determination of the first-formed embryos nevertheless begins soon after the birth of the parent and continues without interruption until after the final moult when the determination of the last-formed embryos is completed. There seems little doubt that the process of determination involves a maternal controlling system which acts upon the embryos at the appropriate stage of development (Lees, 1959). This information can be used in interpreting the present results.

Three classes of parents were observed when the transition to ovipara-production was taking place: (a) parents producing only virginoparae; (b) parents

producing both virginoparae and oviparae; and (c) parents producing oviparae only. When the transition was just beginning, parents from the same group either belonged to category (a) or (b). When it was further advanced they belonged to category (b) or (c). Since the progeny was collected serially in batches it was possible to confirm that with mixed progenies the virginoparae were born first and the oviparae last.

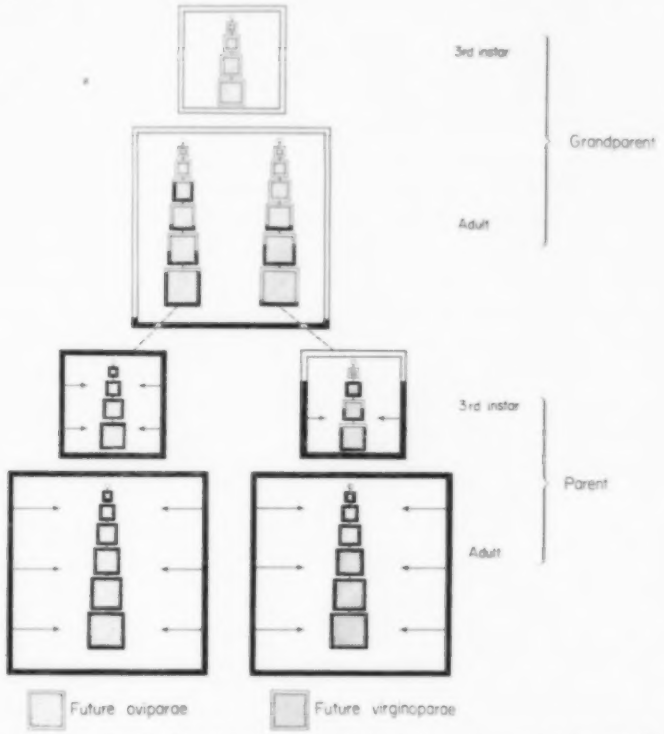


FIG. 6. Diagram illustrating the probable origins of two parents of closely similar age at a time when the ability of the clone to form oviparae is being reinstated. During their embryonic development the two parents are assumed to have occupied corresponding positions in two ovarioles of the grandparent. The remaining ovarioles are omitted. Large and medium-sized rectangles symbolize post-embryonic stages; small rectangles represent embryos. Replacement of the white by a black border indicates that the 'interval timer' has undergone a critical change. It is suggested that this event results in the restoration of the maternal control system regulating embryonic development. Arrows indicate that this system is functioning.

Note that slight differences in the operation of the 'interval timer' result in the parent on the left producing oviparae only, whilst the parent on the right yields both virginoparae and oviparae.

In the case of virginopara-producers (a), it is clear that the maternal function has not yet been restored by the time that all the embryos are determined, i.e. midway through the reproductive period of the parent. With producers of mixed families (b) it seems that the final critical change in the 'interval timer' must have

VOL. 4 1960

supervened during the larval development of the parent when a proportion of the embryos had already been determined as virginoparae. On the other hand, parents belonging to category (c) must have suffered the change during their embryonic development, since at birth the mechanism controlling ovipara-production was fully capable of influencing the first embryos to be formed.

The probable derivation of category (b) and (c) parents is shown schematically in Fig. 6, the assumption being made that parents of similar age have developed synchronously. If the transition can take place in the embryo (as indicated by the occurrence of ovipara-producers), it is not improbable that the transition would also occur at approximately the same time in the soma of the *grandparent*. In this event the latter would become capable of converting to oviparae all those embryos that were not yet determined. As all the parents used were, of course, virginoparae, it seems likely that in ovipara-producers the critical change must often take place during late embryonic development, that is, after the status of the embryo as a virginopara has been decided. Previous work has shown that embryos in this stage of growth occupy positions 4–6 in the embryonic chain (LEES, 1959). However, the existence of clones such as 'A' and 'C' that require several generations to achieve the full sexual response suggests that the transition in the soma of the larval parent sometimes precedes the corresponding transition in the embryos contained in that larva. If the critical change in these embryos is delayed until they, in turn, become part-grown larvae, a succession of generations producing mixed virginoparous and oviparous progeny could arise.

Some of the characteristics of male determination can also be explained in terms of a maternal controlling mechanism which can become fully functional at different times in the ontogeny of the embryo. Table 1 and Figs. 2, 3, and 4 show that both the proportion of parents that are male-producers and the number of males born to each parent are at first abnormally small. In clone 'B', for example, although half the 'first-born' parents in the fifth generation yielded some males, the number per female was only 2, 1, and 1 respectively. In the same clone the generation 6 parents were all male producers, the numbers being 9, 12, 9, 5, 9, and 12. The average of 9·3 males hardly differed from the mean productivity of males (11·6) in a long-established clone under identical environmental conditions (LEES, 1959). Now sex determination appears to take place during the single maturation division of the parthenogenetic egg (SCHWARTZ, 1932). If the capacity to produce males is restored at a time when the parent has already formed some embryos, a reduction in the total of males would be expected. For the transition to have this result it should begin to take effect during the early post-natal development of the parent, since the third or fourth embryos in the ovulation sequence are most likely to be male (LEES, 1959).

TRANSMISSION OF THE 'INTERVAL TIMER' THROUGH AN ALATE GENERATION

Descendants of fundatrix 'D' were used for testing whether the passage of a lineage through an alate parent influenced the rate of operation of the 'interval

timer'. As Fig. 7 shows, the third 'last-born' generation included apterous and alate virginoparae. Five of the apterae were reared in the usual manner, one of them being selected for continuing the lineage. One alata was also reared. It will be noted that the five parents of the 'apterous' line yielded a few males but no oviparae, indicating that the capacity to produce sexual forms was just beginning

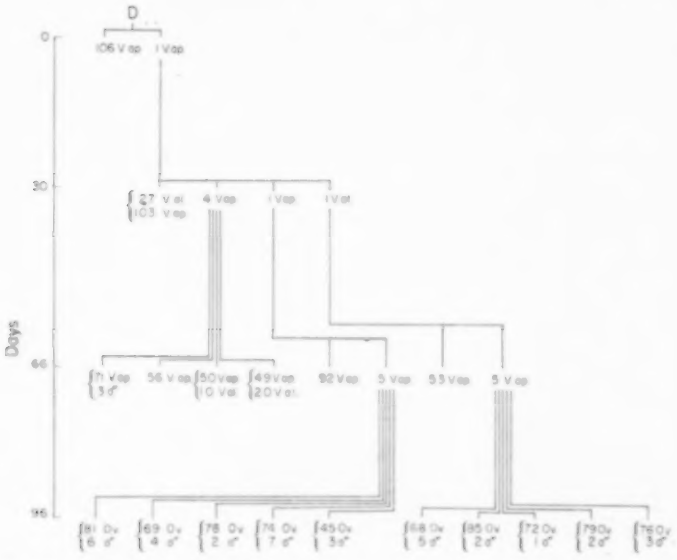


FIG. 7. The genealogy of part of clone 'D' showing the parallel lines of descent through an apterous and an alate virginopara respectively. Note that ovipara-producing parents appear synchronously in the two lines. *V. ap.*, virginoparae apterae; *V. al.*, virginoparae alatae.

to appear. The alata produced no males—a finding which was to be expected, since the tendency of this morph to give rise to sexual forms is always very much less than that of the aptera (LEES, unpublished results). Five fourth-generation apterous parents were then reared in both the 'apterous' and 'alate' lines. Their progeny proved to consist exclusively of males and oviparae. This indicates that the transition had proceeded to completion in both lineages during the 30 days required by this 'last-born' generation.

Similar results were secured in other instances in which the shorter generation time of the 'first-born' lineage was employed. The interpolation of an alate generation therefore appears to have no effect on the 'interval timer'.

THE INFLUENCE OF TEMPERATURE ON THE PRODUCTION OF SEXUALES

Similar experiments were conducted under short day conditions at 20 and 11°C, 5–7 fundatrices being reared at each temperature. The progeny was again

used to establish 'first-born' and 'last-born' lines. But only one parent was subsequently tested in each generation.

The descendants of three fundatrices exposed permanently to a 12-hr day at 20°C are shown in Table 2. The results again indicate that the development of sexuales is not associated with any particular generation. Thus among 'last-born' aphids, oviparae were first encountered in the third and fourth generations, whereas

TABLE 2—PROGENIES RECORDED IN THREE OF THE CLONES EXPOSED FROM THE FUNDATRIX GENERATION TO A 12-HR DAY AT 20°C

(V., virginoparae; Ov., oviparae; ♂, males)

Fundatrix (gen. 1) and progeny (gen. 2)	Days	9	20	25	32	44	45
	Gen. no. 'First-born'	3	4		5	6	
	Gen. no. 'Last-born'			3			4
'G' 122 V.	'First-born'	114 V.	91 V.		22 V. 41 Ov. 16 ♂	10 V. 86 Ov. 15 ♂	
	'Last-born'			97 V. 14 Ov.			79 Ov. 12 ♂
'H' 59 V.	'First-born'	59 V.	53 V. 7 ♂		43 V. 2 ♂	16 V. 48 Ov. 7 ♂	
	'Last-born'			40 V. 6 Ov. 6 ♂			39 V. 8 Ov. 4 ♂
'I' 87 V.	'First-born'	69 V.	135 V.		82 V. 16 ♂	14 V. 71 Ov. 23 ♂	
	'Last-born'			21 V.			56 Ov. 11 ♂

'first-born' aphids failed to develop oviparae until the fifth and sixth. The initial occurrence of oviparae in the two lineages was again approximately synchronous, the precise time of the 'first appearance' often depending on the availability of a test generation at the critical moment, rather than on intraclonal variations. This

has again been responsible for the fact that oviparae were found in the 'last-born' lineages of clones 'G' and 'H' slightly before they were recorded in the 'first-born' lineage.

The most striking feature of the response at 20°C is that the time lapse between the onset of reproduction in the fundatrix and the same event in the first ovipara-producers is relatively short, amounting to only 25 days in clones 'G' and 'H'. The transition in clone 'I' required a little longer, but was complete by 45 days (Table 2).

Results at this temperature provide further evidence that the production of males is governed by an 'interval timer' working independently of generation (see clone 'H', for example). The incidence of males in clones 'H' and 'I' shows that this form usually appears before the oviparae. Nevertheless, the order of appearance seems sometimes to be reversed, for in clone 'G' the first ovipara-producing family (generation 3 in the 'last-born' lineage) contained no males even though the

TABLE 3—PROGENIES RECORDED IN THREE OF THE CLONES EXPOSED FROM THE FUNDATRIX GENERATION TO A 12-HR DAY AT 11°C

(V,. virginoparae; Ov., oviparae; ♂, males)

	Days	25	45	66	95	114	145
Fundatrix (gen. 1) and progeny (gen. 2)	Gen. no. 'First-born'	3		4		5	
	Gen. no. 'Last-born'		3		4		5
'J' 104 V.	'First-born'	97 V.		18 V.		1 V. 3 Ov.	
	'Last-born'		10 V.		6 Ov.		
'K' 50 V.	'First-born'	97 V.		40 V.		21 Ov.	
	'Last-born'		39 V.		9 Ov.		
'L' 84 V.	'First-born'	42 V.		23 V.		9 Ov.	
	'Last-born'		3 V.		14 V.		18 Ov.

progeny should have been sufficiently numerous for demonstrating an incipient or fully developed capacity for male-production.

Table 3 shows that at 11°C the interval from fundatrix to ovipara-producing generation was much longer than at 15°C, occupying 66–95 days in the case of

clones 'J' and 'K' and between 95 and 114 days in clone 'L'. The appearance of oviparae was again independent of generation. No males were formed at 11°C. As previously mentioned (p. 158), this is probably due to the low fecundity of the later generations.

The 'first appearance' of sexuales at the three temperatures is summarized in Table 4. The time limits, which record the performances of the 'fastest' and 'slowest' clones, serve to emphasize the comparative constancy of this factor in the two lineages, despite the clonal variation and the difficulties of precise time measurement. A further point is noteworthy. If the 'first-born' or 'last-born' lineages are inspected separately, it becomes evident that a given sexual form—ovipara or male—appears in virtually the same generation irrespective of temperature. For

TABLE 4—THE INFLUENCE OF TEMPERATURE ON THE TIME INTERVAL WHICH PRECEDES THE FIRST APPEARANCE OF OVIPARA-PRODUCING PARENTS (A) AND MALE-PRODUCING PARENTS (B) WHEN YOUNG CLONES ARE EXPOSED CONTINUOUSLY TO A 12-HR DAY

(The limits refer to the performance of the 'fastest' and 'slowest' clones)

	Temp. °C	'First-born' lineage		'Last-born' lineage	
		Days	Gen. no.	Days	Gen. no.
A	20	32–44	5–6	25–45	3–4
	15	55–76	5–6	66–96	4–5
	11	114	5	95–145	4–5
B	20	20–32	4–5	25–45	3–4
	15	35–76	4–6	30–66	3–4
	11	no ♂♂	—	—	—

instance, oviparae were first found in the fifth or sixth 'first-born' generation at all three temperatures. This must mean that the temperature coefficient of the 'interval timer' is roughly the same as those of the growth processes that collectively occupy the generation time.

THE INFLUENCE OF AN INITIAL LONG-DAY RÉGIME ON THE ABILITY TO FORM SEXUALES

Nineteen fundatrices and their descendants were placed on a daily light schedule of 16 hr at 15°C. Some clones were soon lost, owing to their infertility, but ten were reared for ten generations in long days, one parent being saved in each generation for continuing the lineage. In this experiment no attempt was made to select the prospective parents from the same point in the sequence of progeny. The generation time was therefore irregular. In the eighth generation, which was reached after 132 days, one daughter virginopara again received long-day treatment whilst a second individual was exposed to a short 12-hr day from the first instar. The effect of this régime on one representative lineage (clone 'M') is summarized in Table 5.

The initial exposure to long days was considerably longer than the 55–96 day period which is normally required for the 'interval timer' to operate at 15°C. It is therefore interesting to note that the capacity to form oviparae has in fact fully developed by 132 days, since the short-day parents yielded only oviparae.

TABLE 5—PROGENIES RECORDED IN CLONE 'M' DURING EXPOSURE TO LONG-DAY CONDITIONS FOR TEN GENERATIONS. NOTE THAT AN EXTRA EIGHTH-GENERATION PARENT WAS REARED IN SHORT DAYS TO TEST THE ABILITY TO FORM OVIPARAE

(V., virginoparae; Ov., oviparae; ♂, males)

Photoperiod (hr)	Days	Gen. no.	Progeny
16	0	2	59 V.
16	30	3	80 V.
16	49	4	127 V.
16	70	5	92 V. 5 ♂
16	91	6	72 V. 14 ♂
16	117	7	85 V. 15 ♂
16	132	8	59 V. 11 ♂
12	152	9	57 Ov. 9 ♂
16	152	9	49 V. 14 ♂
16	173	10	48 V. 14 ♂

No males were produced in clone 'M' in the three generations following the fundatrix. But, in contrast to ovipara-production, this function does not remain latent indefinitely in long days. In clone 'M' the first males appeared in generation 5 after a time-interval of 70 days (Table 5). The numbers were small at first, but by the next generation the male-forming capacity had completely developed. Allowing for normal variation (see LEES, 1959), the full complement of males was then produced in every generation.

The other nine clones also yielded males during the protracted exposure to a long photoperiod. In this respect the behaviour is similar to that of a long-established parthenogenetic clone which, as has been shown previously (LEES, 1959), produces similar numbers of males irrespective of photoperiod. Despite

VOL. 4 1960

this resemblance, however, there seems no doubt that the ability to form males is acquired later than it is under conditions of short photoperiod. In the ten clones examined, only three were producing males after 70 days. Subsequently the number of male-producers reached five after 91 days, seven after 117 days, nine after 132 days, and ten after 152 days. Clones 'B'–'F', which received a permanent 12-hr photoperiod, responded more promptly, since the generations falling at 55, 66, 76, and 96 days included 20, 47, 68, and 100 per cent of male-producers respectively.

DISCUSSION

The results presented in this paper show that viviparae from young clones of *Megoura* cannot be induced to form sexuales, whereas suitable environmental stimuli readily elicit this response in older clones. BONNEMAISON (1951) employed the expression 'facteur fondatrice' to describe this phenomenon in *Brevicoryne brassicae*. Since, however, the mechanism responsible for this refractory period is essentially a device for measuring time, a term which embodies this notion is preferable. It is also possible that the physiological 'clock' is set in motion in the fertilized egg and not in the fundatrix. Although 'interval timer' has been defined by PITTENDRIGH (1958) in terms of 24-hr rhythms, it seems appropriate to apply it also to longer periods of time measurement.

Some of the characteristics of the timing mechanism have been demonstrated. It is not immune to environmental agents since its functioning is markedly temperature-dependent. In this respect it is worth noting that temperature has different and contrasting effects on virginoparae from young and old clones. In the first instance high temperature shortens the interval before the aphids are capable of producing sexuales in response to short photoperiods; in the second instance high temperature opposes the action of short days and thus *prevents* the appearance of sexuales. There is little doubt that temperature is here affecting two quite different processes.

In contrast, there is much less evidence that the length of the refractory period is influenced by photoperiod. Thus male-producing virginoparae develop in all day lengths, although there are signs that the event is more delayed if the clone has first been exposed to a long photoperiod. The aphids also acquire the ability to form oviparae under all conditions of photoperiod, although this potentiality remains unrealized under long-day régimes. Such experiments show that the aphids do not require a period of short-day treatment before these functions can be restored. This seems to contrast with the condition described in birds in which exposure to long days results in the maintenance of the post-breeding refractory condition, the gonads remaining unaffected by long-day photostimulation until an adequate period of short-day treatment has been experienced (WOLFSON, 1952).

The possible nature of the innate 'interval timer' is of considerable interest. Classical accounts of parthenogenesis in aphids state that the formation of a female egg involves a single non-reductional division of the chromosomes which leaves

the egg with the full diploid set (MORGAN, 1910; SCHWARTZ, 1932). As a similar maturation process doubtless occurs in all the parthenogenetic morphs, all types of female should possess the same chromosome number. And if these forms are part of a single clone, complete identity of hereditary nuclear material might also be expected. These considerations suggest that progressive morphological or physiological changes are mainly controlled through the cytoplasm, as suggested by WILSON (1938). This does not of course exclude the possibility that these cytoplasmic elements interact with the nuclear genes.

The controlling mechanism seems to possess unique features, the property of transformation with time being apparently combined with a capacity for self-replication. This is inferred from the many cell divisions which are interposed between the fertilized egg and the somatic cells of the first male- or oviparae-producer. In each generation there are the cell divisions associated with cleavage, blastoderm formation, the growth of the 'germ cylinder', and the differentiation of the definitive gonads (UICHANKO, 1924). And several generations always supervene before sexuales in fact appear. Since there is also abundant evidence that the 'interval timer' does not require a fixed number of generations to operate, it is virtually certain that it is unaffected by the number of cell divisions.

VOL. 4 1960

This argument suggests that it would be unrealistic to picture a cytoplasmic substance which was subjected to attenuation by cell division until falling below a critical threshold. On the contrary, the propagation of the effect through very many cell generations indicates that the mechanism may be capable of self-duplication, like a plasmagene, while suffering a continuous temperature-dependent change. This fundamental cellular transformation is evidently unaffected by the type of parent (aptera or alata) of which the cells form a part.

It is uncertain at present whether one should think of a single cytoplasmic 'interval timer' or of several with different rate characteristics. The determination of males and oviparae appears to involve entirely different physiological processes. The first, which may involve chromosome loss in the maturing ovum, is influenced by environmental temperature but not by photoperiod. On the other hand, the fate of the ovipara is decided much later when the embryo is half-grown—perhaps by a maternal hormone. The latter process is influenced both by temperature and photoperiod (LEES, 1959). These differences suggest that there may be two separate 'interval timers', one for the male and the other for the ovipara. If this were true, there is no particular reason why both should operate at precisely the same rate—a circumstance which might account for the earlier appearance of males in young clones. However, the evidence is by no means conclusive, and there are even slight indications from the study of clonal variation that there are links between the two processes. It also remains to be decided whether the morphological changes which occur in the descendants of fundatrices are also governed by an 'interval timer' which transcends generation.

Very little is known of the physiological changes which supervene when the 'time interval' has elapsed. BONNEMAISON (1951) has suggested that when the 'facteur fondatrice' has run its course, it is supplanted by a 'facteur interne' which

promotes the development of the sexual morphs.* The 'facteur interne' can in turn be inhibited by various environmental agents, such as a long photoperiod or high temperature. BONNEMAISON also maintains that this state does not persist indefinitely in lines that reproduce continuously by parthenogenesis for a year or more. *Brevicoryne brassicae* was one species in which the 'facteur interne' could be dissipated in this manner, the resulting anholocyclic strains producing sexuales sparingly or not at all.

A somewhat different interpretation can be offered for *Megoura*. It is known that in old clones of this species the environment acts primarily on the various maternal 'switch' mechanisms and that these in turn direct the egg or embryo into the appropriate developmental paths. The failure of virginoparae from young clones to produce sexuales can be accounted for in two ways: the first possibility is that the maternal switch mechanisms are functioning normally, while the embryos are, for some reason, incapable of responding; the second, and more probable, explanation is that there are 'defects' in these mechanisms, the outcome being that all eggs become female and all embryos virginoparae. According to the latter view, the operation of the 'interval timer' results in the restitution of these mechanisms, so that responsiveness to the environment is restored. Whether the environmental agents in question have an inhibitory or a promotive action cannot be judged until more direct experimental evidence is forthcoming. It has been pointed out elsewhere that the ability of *Megoura* to form sexuales has remained unimpaired during 10 years of parthenogenetic reproduction (LEES, 1959). In this it resembles the clone of *Sappaphis plantaginea* recently examined by BONNEMAISON (1958).

SUMMARY

(1) Young clones of *Megoura viciae* produce only virginoparae when exposed to a short (12 hr) photoperiod; the capacity to form sexuales (males and oviparae) appears later as the clone ages. In investigating this response, 'first-born' and 'last-born' lineages were set up by breeding from an early and a late offspring in each generation.

(2) The appearance of sexuales depends on the passage of time and not on the number of generations interposed between fundatrix and sexuales-producer. For example, at 20°C ovipara-producing virginoparae developed synchronously in the two lineages after a time-interval (from the fundatrix) of 25–45 days. By then the 'first-born' lineages of the various clones had passed through five or six generations, whilst the 'last-born' had passed through only three or four. Differences in the time-interval are due to inter-clonal rather than to intra-clonal variation.

(3) The production of the first males is also unconnected with the generation number, but the 'time-interval' is rather shorter than for ovipara-production.

* It should be noted that BONNEMAISON (1951) also uses the term 'facteur fondatrice' to describe other functions, e.g. the effect of clonal age on the production of alatae under conditions of high population density. 'Facteur interne' has also been invoked to explain more than one type of phenomenon.

(4) In young clones that are just beginning to yield sexual forms, the number of males per parent is usually low; and ovipara-producers often give birth to a series of virginoparae, followed by oviparae. It appears that the maternal faculty for producing these morphs may be restored when a proportion of the eggs and embryos are already determined as females and virginoparae.

(5) The 'interval timer' mechanism is temperature-dependent, the positive coefficient approximating to that of growth itself.

(6) The rate of operation of the 'interval timer' is not affected if winged virginoparae are used as parents in the place of apterae.

(7) When the fundatrices and their descendants are exposed continuously to a 16-hr photoperiod, the capacity to form oviparae remains latent indefinitely. But after the 'time-interval' has elapsed, the normal response to a 12-hr photoperiod can be elicited at any time. In contrast, male-producing virginoparae eventually appear in constant long days.

(8) As nuclear uniformity would be expected within a clone, it is suggested that the timing mechanism is located in the cytoplasm. Since, also, its operation appears to be independent of cell division, the elements involved may be capable of self-duplication while suffering a continuous temperature-dependent transformation. This cytoplasmic change evidently culminates in the restitution of the maternal control system which permits the eggs and later, the embryos, to develop along alternative pathways in response to the appropriate environmental signals.

VOL. 4 1960

REFERENCES

BONNEMAISON L. (1949) Sur l'existence d'un facteur inhibitant l'apparition des formes sexuées chez les Aphidinae. *C. R. Acad. Sci., Paris* **229**, 386–388.

BONNEMAISON L. (1951) Contribution à l'étude des facteurs provoquant l'apparition des formes ailées et sexuées chez les Aphidinae. *Ann. Epiphyt.* (C) **2**, 1–380.

BONNEMAISON L. (1958) Facteurs d'apparition des formes sexupares ou sexuées chez le puceron cendré du pommier (*Sappaphis plantaginea* Pass.). *Ann. Epiphyt.* (C) **3**, 331–355.

LEES A. D. (1959) The role of photoperiod and temperature in the determination of parthogenetic and sexual forms in the aphid *Megoura viciae* Buckton—I. The influence of these factors on apterous virginoparae and their progeny. *J. Ins. Physiol.* **3**, 92–117.

MARCOVITCH S. (1924) The migration of the Aphididae and the appearance of the sexual forms as affected by the relative length of daily light exposure. *J. agric. Res.* **27**, 513–522.

MORGAN T. H. (1910) The chromosomes in the parthenogenetic and sexual eggs of phylloxerans and aphids. *Proc. Soc. exp. Biol., N.Y.* **7**, 161–162.

PITTENDRIGH C. S. (1958) Perspectives in the study of biological clocks. *Symposium on Perspectives in Marine Biology*, pp. 239–266. Univ. of California Press, Berkeley.

SCHWARTZ H. (1932) Der Chromosomzyklus von *Tetraneura ulmi* de Geer. *Z. Zellforsch.* **15**, 645–687.

UICHANKO L. B. (1924) Studies on the embryology and postnatal development of the Aphididae with special reference to the 'symbiotic organ' or 'mycetome'. *Philippine J. Sci.* **24**, 143–247.

WILSON F. (1938) Some experiments on the influence of environment upon the forms of *Aphis chloris* Koch (Aphididae). *Trans. R. ent. Soc. Lond.* **87**, 165–180.

WOLFSON A. (1952) The occurrence and regulation of the refractory period in the gonadal and fat cycles of the junco. *Anat. Rec.* **113**, 42.

. *Ins. Physiol.*, 1960, *Vol.* 4, *pp.* 176 *to* 183. *Pergamon Press Ltd., London. Printed in Great Britain*

PLASTRON RESPIRATION IN THE EGGS OF BLOWFLIES

H. E. HINTON

Department of Zoology, University of Bristol

(*Received* 15 *October* 1959)

Abstract—In the egg-shells of the blowflies *Calliphora* and *Lucilia* a continuous film of air is held between vertical columns in the inner part of the chorion. This film of air is continuous with that held in the median area between the hatching lines. The chorion of the median area between the hatching lines consists of three distinct layers. The outer and inner layers consist of struts or columns that arise perpendicularly from the middle layer. A continuous film of air is held between the columns of each layer. Holes through the middle layer of the chorion effect the continuity of the air films of the outer and inner layers. The columns of the inner layer are connected to a thin and continuous inner sheet of chorion. The columns of the outer layer are branched at their apices in a plane normal to their long axes. These horizontal branches form a fine and open hydrofuge network that provides a large water–air interface when the egg is immersed.

The film of air held in the outer layer of the area between the hatching lines functions as a plastron when it rains or the egg is otherwise immersed in water. The eggs of blowflies are normally laid on decomposing materials, and the surface tension of water in contact with these is much lowered by surface active substances. The resistance of the plastron to hydrostatic pressures varies directly as the surface tension of the liquid. These considerations would seem to provide an explanation for the fact that the plastrons of blowflies not only resist wetting in clean water by hydrostatic pressures far greater than any to which they are at all likely to be exposed in nature, but, paradoxically, are more resistant to pressure than are the plastrons of some wholly aquatic insects that live in clean water.

INTRODUCTION

THE eggs of many terrestrial insects are glued to the substrate in sites where they are necessarily submerged beneath a layer of water when it rains heavily. To be covered by water for several hours or even days, a time that may exceed the incubation period, is not a very exceptional circumstance but is a normal hazard of their environment. When the environment of these eggs is seen as one that is alternately dry and flooded, it is perhaps not surprising to find that many of them have respiratory structures previously thought to be restricted to aquatic insects. It has recently been shown that the terrestrial eggs of many families of the Diptera-Cyclorrhapha (Dryomyzidae, Sepsidae, Sphaeroceridae, Drosophilidae, Cordiluridae, Muscidae) have a plastron (HINTON, 1959, 1960a, b). In this paper the structure of the respiratory system and plastron of the egg-shells of *Calliphora erythrocephala* Meig. and *Lucilia sericata* Meig. is described.

STRUCTURE OF RESPIRATORY SYSTEM OF EGG-SHELL OF *CALLIPHORA*

The dimensions and general appearance of the egg of *Calliphora erythrocephala* are shown in Fig. 1. The whole of the surface except for the area between the

hatching lines has a thin layer of cement produced by the accessory glands. The hatching lines are on the middle of the dorsal surface. They extend from about the posterior eighth to very near the micropyle, and throughout most of their

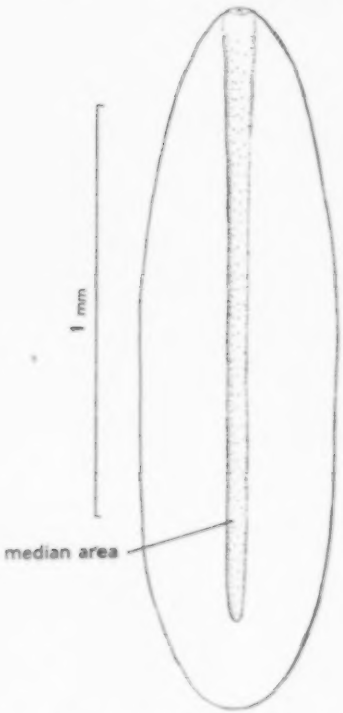


FIG. 1. *Calliphora erythrocephala.* Dorsal view of egg.

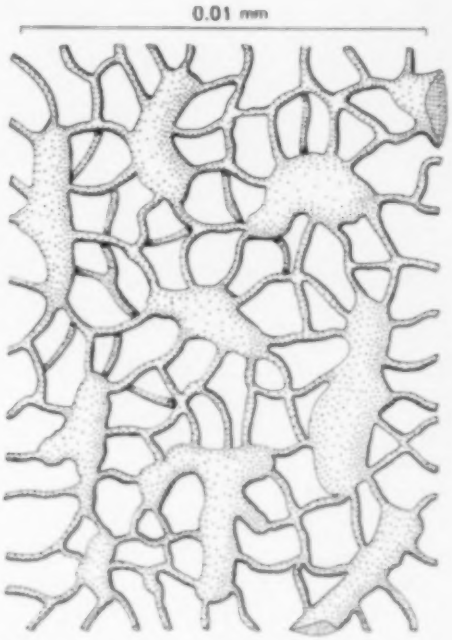


FIG. 2. *Calliphora erythrocephala.* Plastron network of median area between hatching lines.

length are more or less parallel. The chorion everywhere except between the hatching lines consists of a relatively thick outer sheet connected to a thin inner sheet by vertical columns (Figs. 3, 4). A continuous layer of air is present between

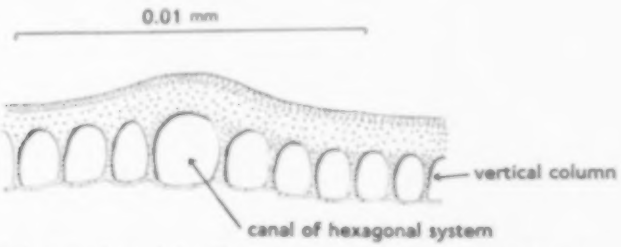


FIG. 3. *Calliphora erythrocephala.* Transverse section of a part of the dorso-lateral chorion.

the vertical columns. The vertical columns are arranged in somewhat irregular hexagons, the boundaries of which correspond to the boundaries of the follicular cells. At the boundaries of the hexagons the vertical columns are absent, so that there is here a continuous hexagonal network of air canals. The vertical columns

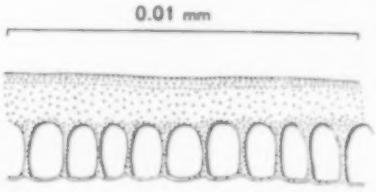


FIG. 4. *Lucilia sericata*. Transverse section of a part of the dorso-lateral chorion.

supporting these canals are slightly wider apart than those elsewhere, and the outer layer of the chorion is here more deeply excavated: in section, therefore, the canals of the hexagonal system are easily recognizable as distinctly higher and wider spaces (Fig. 3). The chorion above the canals of the hexagonal system is feebly convex so that the shell has a superficial hexagonal pattern of low ridges that is widely separated from but directly above the hexagonal pattern of air canals.

It has previously been shown that in the higher Diptera the inner sheet of chorion may be continuous or may be an open network, as it is in the Muscids of the subfamily Muscinae (HINTON, 1960b). In *Calliphora* it is a continuous sheet. Paraffin oil applied to any part of its inner surface does not displace the air between the vertical columns.

The structure of the chorion between the hatching lines is very different from that elsewhere. It consists of an outer and an inner layer, each of which holds a continuous film of air. Between these two layers there is a thick middle layer. Holes or aeropyles through the middle layer effect the continuity of the outer and inner films of air. The outer layer consists of struts that arise perpendicularly from the outer surface of the middle layer (Fig. 5). The struts are widened towards their apices and are apically branched in a plane normal to their long axes. The horizontal branches connect the apices of adjacent struts and also often anastomose with one another (Fig. 2). They form a fine and open hydrofuge network at the surface of the median area. When the egg is immersed in water, the water–air interface of the plastron is confined to the interstices of the surface network of the median area. When the egg is not covered by water, the interstices of the outer network provide a direct route for entry of gases into the outer and inner layers of the median area.

The inner layer of the median area consists of columns that extend vertically inwards from the inner surface of the middle layer and are joined to a thin and continuous inner sheet of chorion. The vertical columns of the inner layer are shorter but otherwise similar to those of the chorion outside the hatching lines, and

this layer also has an hexagonal pattern of air canals similar to that of the chorion outside the hatching lines.

Paraffin oil applied to the inner surface of the inner sheet of the median area does not displace the air between the vertical columns. However, paraffin oil applied to any part of the outer network of the median area displaces the air in the outer layer, runs through the aeropyles and displaces the air between the vertical columns, and, if enough is applied, runs out of the median area and displaces the air between the vertical columns elsewhere in the chorion.

VOL. 4 1960

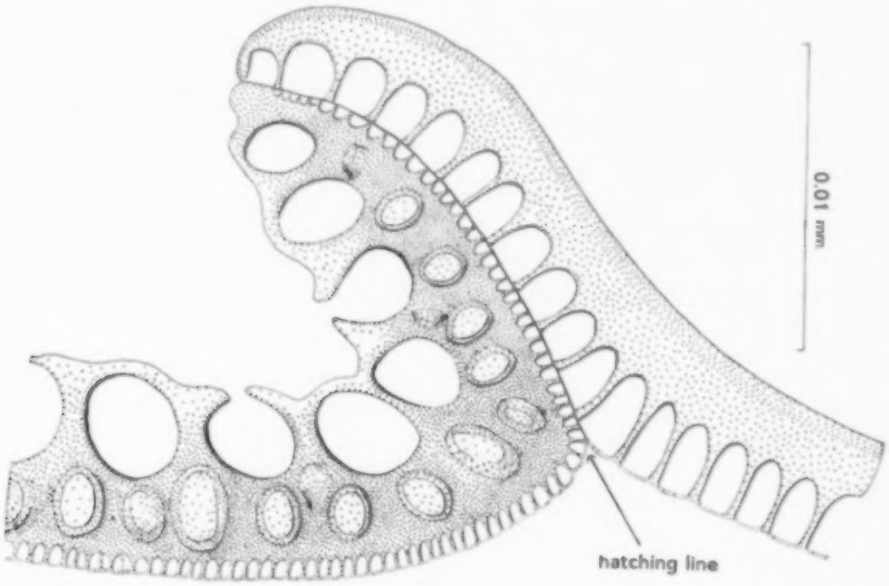


FIG. 5. *Calliphora erythrocephala.* Transverse section through the right side of the median area between the hatching lines.

As may be seen from Fig. 5, the hatching line on each side extends between the apices of the vertical columns of the median area and the apices of the vertical columns of the part of the chorion contiguous with the median area. The apices of the vertical columns of the two structures are here joined to an open network in the plane of the hatching line and not to a continuous sheet as they are elsewhere. It is because the continuous inner sheet is replaced by an open network in the plane of the hatching line that oil so rapidly invades the spaces between the vertical columns of all parts of the shell when applied in sufficient quantity to the outer network of the median area.

When the larva hatches, it forces apart the inner network of the median area and the contiguous inner network of the chorion outside the median area. The hatching line is thus a line of weakness that results from the two networks being

relatively lightly stuck together. On hatching, either the right or the left hatching line is split along its anterior third or so, and the split of the other hatching line is usually confined to its extreme anterior end. When the eggs are laid in batches, an egg will sometimes have others stuck across its hatching lines, and the larva may escape by making a hole through the ventral surface of the shell. DAVIES (1950) claims that the chorion usually ruptures on hatching along the outer margin of either of what he calls the hatching-pleats, and that it is not necessary to postulate a line of weakness here. However, the hatching line or line of weakness runs along the centre of the hatching-pleats (Fig. 5), and I have never seen a dorsal split that was not a split of the hatching line.

The structure of the respiratory system of the egg-shell of *Lucilia sericata* Meig. is identical in all essential particulars to that of *Calliphora erythrocephala*: I have been unable to find any constant structural differences between the two species.

RESISTANCE OF THE PLASTRON TO EXCESS PRESSURES

The term plastron has been restricted to describe a gas film of constant volume and an extensive water–air interface. Such films are held in position by a system of hydrofuge structures that resist the entry of water under pressure. In well-aerated water a plastron enables an insect to remain immersed indefinitely, when it obtains the oxygen it requires from the ambient water.

A column of water was used to study the resistance of the plastron to an hydrostatic pressure of 7 cm Hg. Its resistance to higher pressures was tested in a chamber connected to a mercury reservoir by rubber tubing. The pressure required was obtained by raising the reservoir the appropriate distance above the chamber. Live eggs were used in all experiments. The figures for the resistance of the plastron to excess pressures are therefore minimum figures, since any oxygen uptake by the egg must reduce the back pressure of the system. The maximum possible reduction due to respiration would be equivalent to a rise in the hydrostatic pressure of about 16 cm Hg. However, as in all experiments the total volume of the eggs used was small in comparison to that of the well-aerated tap water in the chamber, and most experiments lasted only $\frac{1}{2}$ hr, it is probable that the actual pressures were only a few cm greater than those recorded. During the experiments in which the eggs were subjected to excess pressures for 24 hr, a small number either hatched or split one or other of the hatching lines. These eggs are not entered in Table 1, since the plastron is often more easily wetted from the exposed face of the hatching line. For instance, it has been shown that in the egg-shell of *Scopeuma stercorarium* L. the interstices of the face of the hatching line are larger than those of the plastron surface, and any splitting of the hatching line results in the plastron being wetted by pressures lower than those ordinarily required to wet it when the hatching lines are intact (HINTON, 1960a).

The plastron of *Lucilia sericata* Meig. appears to be as resistant to excess pressures as is that of *Calliphora*. In experiments at 100 cm Hg for 30 min the percentage retaining over 90 per cent of the plastron varied from 21 to 50, and in

VOL.
4
1960

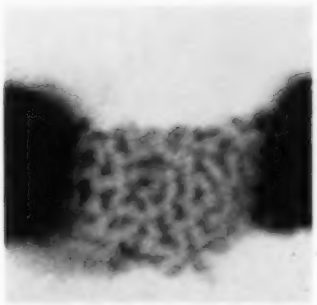


FIG. 6. *Calliphora erythrocephala*. Photograph of a surface view of the plastron network of the median area between the hatching lines.

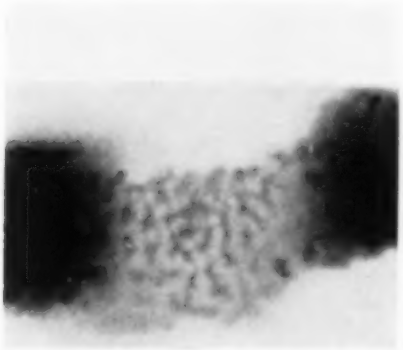


FIG. 7. *Calliphora erythrocephala*. Photograph of a surface view of the plastron network of the median area between the hatching lines. In Figs. 6–7 the horizontal branches connecting the dilated apices of the perpendicular struts are discernible in some areas.

FIG. 8. *Calliphora erythrocephala*. Photograph of a transverse section through the median area between the hatching lines at the middle of its length.

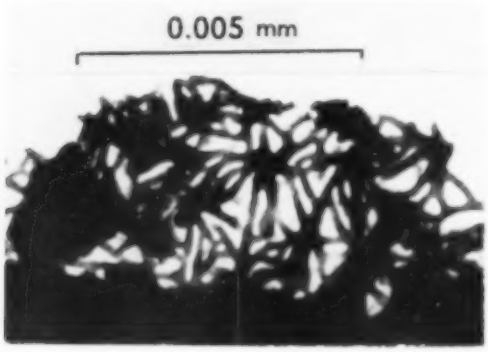


FIG. 9. *Simulium variegatum*. Electron micrograph of the plastron of the spiracular gill.

one experiment at 1 atm for 30 min, 80 per cent of the eggs retained over 90 per cent of the plastron.

The plastrons of blowfly eggs resist wetting by hydrostatic pressures considerably greater than any to which they are at all likely to be exposed in nature (Table 1).

TABLE 1—EFFECTS OF EXCESS PRESSURES UPON THE RETENTION OF THE PLASTRON OF *Calliphora erythrocephala* (19–21°C)

Number	Pressure (cm Hg)	Time (hr)	% retaining over 90% of plastron
13	7	24	92
10	14	24	80
11	30	½	100
10	60	½	90
10	76	½	100
10	76	½	100
11	76	½	100
11	76	½	72
10	100	½	70
10	100	½	60
10	100	½	50
10	100	½	50
10	100	½	20
11	100	½	18
11	152	½	27
10	152	½	20
10	152	½	0
4	152	½	0
11	152	½	0
9	152	½	0

Moreover, they are more resistant to wetting by excess pressures than are the plastrons of some aquatic insects, e.g. *Taphrophila vitripennis* Meig. (HINTON, 1957, Table 7). It has previously been suggested (HINTON, 1960a) that the probable explanation of the paradox that the plastrons of many terrestrial eggs are more resistant to excess pressures in clean water than are the plastrons of some wholly aquatic insects that live in streams is to be found in the fact that eggs laid in decomposing organic matter are often exposed to concentrations of surface active substances that rarely if ever occur in streams. For instance, using a DuNoüy ring balance it was found that the surface tension of the temporary pools of rain water on cow pats is reduced to about 50 dyn/cm, whereas under comparable conditions the surface tension of water standing on decomposing flesh is reduced to about 40 dyn/cm. Dilution has relatively little effect on surface tension, e.g. when the liquid collected from the surface of a cow pat was diluted with tap water to a quarter of its strength its surface tension was only increased to 55·5 dyn/cm. The resistance of the plastron to pressures varies directly as the surface tension of the liquid. Any change in the geometry or the nature of the surface of the plastron

network that increases its resistance to wetting by surface active substances also increases its resistance to wetting in clean water by excess pressures, since wetting by excess pressures always occurs before there is a mechanical breakdown of the plastron meshwork. Therefore selection for greater resistance to wetting by surface active substances inevitably results in greater resistance to wetting by excess pressures in clean water.

The eggs of flies can be placed in two groups according to the resistance of their plastrons to excess pressures: (a) the species that lay in dung, especially cow dung, and (b) the species that lay in decomposing fungi and flesh. Under natural conditions there would appear to be no great difference in the maximum depths of water to which the two groups of eggs may from time to time be subjected, and it has been suggested (HINTON, 1960a) that in so far as differences in the resistance of the plastron to wetting are related to outward circumstance, they are related to environmental differences in surface active substances rather than hydrostatic pressures. In the first group the resistance of the plastron to wetting falls off rapidly on exposure to excess pressures of over 30–40 cm Hg for 30 min, whereas the second group resist excess pressures of 60–100 cm for 30 min or more. In this connexion the Sepsidae are of particular interest. They lay both in dung and liquefying flesh, and the resistance of their plastrons to excess pressures is distinctly greater than any of the species restricted to dung (HINTON, 1960a). As might be expected, *Calliphora* and *Lucilia* belong to the second group.

DISCUSSION

The only previous attempt to give a detailed account of the respiratory structures of the egg-shells of blowflies appears to be that of WIGGLESWORTH and BEAMENT (1950), who worked on *Calliphora erythrocephala*. They relied chiefly on the cobalt sulphide injection method. As has been previously noted (HINTON, 1959), it is generally quite difficult to fill the air spaces in egg-shells with cobalt sulphide. When the eggs are lightly injected, the sulphide adheres to the surfaces of the plastron network and elsewhere to the surfaces of the vertical columns and the tops and bottoms of the air spaces. Thus at first sight it can appear that these solid structures are themselves impregnated with sulphide, which is what WIGGLESWORTH and BEAMENT believe. For instance, they claim that in the shell of *Calliphora* the air is contained within the vertical columns and within the inner sheet of the chorion and not in the spaces between the columns. They are thus left with the problem of accounting for the spaces between the columns, that is, the true air spaces. The spaces between the columns are said by them to be filled with a glassy material which is 'mainly water'.

The air spaces in insect egg-shells are, as one would expect them to be, considerably wider than the mean free path of the oxygen molecule. When the columns or struts supporting the air spaces are examined with the electron microscope, it immediately becomes clear that they are not 'spongy' at the necessary level. Electron micrographs having a resolution of about 100 Å of the struts of the egg-shell of *Dryomyza* show no holes or cavities (HINTON, 1960a).

WIGGLESWORTH and BEAMENT (1950, p. 447) state that, "Aeration or 'pneumatization' of the cavities in the chorion does not seem to take place in the normal *Calliphora* egg . . ." The oviposition of large numbers of *Calliphora* and *Lucilia* eggs has been observed. In every instance the chorion was seen to be air-filled when the egg left the ovipositor. Several individuals of each species were dissected, and the mature eggs in the common oviduct were always filled with air. Aeration or 'pneumatization' in *Calliphora* and *Lucilia* occurs while the egg is still bathed in fluid in the common oviduct. In this respect blowfly eggs do not differ from those of all other oviparous Diptera-Cyclorrhapha that have been examined, e.g. species of Syrphidae, Dryomyzidae, Sepsidae, Sphaeroceridae, Drosophilidae, Cordiluridae, and Muscidae.

The plastron network on the surface of the median area between the hatching lines has been overlooked by WIGGLESWORTH and BEAMENT. It is of some interest to note that the plastrons of *Calliphora* and *Lucilia*, and those of other dipterous eggs, are superficially very similar in structure to the plastrons of the spiracular gills of the pupae of the Simuliidae (Fig. 9).

VOL. 4 1960

Acknowledgement—I am grateful to Miss ELAINE HARRIS for taking the electron micrograph.

REFERENCES

DAVIES L. (1950) The hatching mechanism of Muscid eggs (Diptera). *J. exp. Biol.* **27**, 437–445.

HINTON H. E. (1957) The structure and function of the spiracular gill of the fly *Taphrophila vitripennis*. *Proc. roy. Soc.* (B) **147**, 90–120.

HINTON H. E. (1959) Plastron respiration in the eggs of *Drosophila* and other flies. *Nature, Lond.* **184**, 280–281.

HINTON H. E. (1960a) The structure and function of the respiratory horns of the eggs of some flies. *Phil. Trans.* **243**, 45–73.

HINTON H. E. (1960b) The chorionic plastron and its role in the eggs of the Muscinae (Diptera). *Quart. J. micr. Sci.* In press.

WIGGLESWORTH V. B. and BEAMENT J. W. L. (1950) The respiratory mechanisms of some insect eggs. *Quart. J. micr. Sci.* **91**, 429–452.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 184 *to* 189. *Pergamon Press Ltd., London. Printed in Great Britain*

THE RESPIRATORY STRUCTURES IN THE EGGS OF HIGHER DIPTERA

V. B. WIGGLESWORTH and J. W. L. BEAMENT

The Agricultural Research Council Unit of Insect Physiology, Department of Zoology, Cambridge

(*Received* 11 *November* 1959)

Abstract—An injection mixture consisting of equal parts olive oil and kerosene, which is followed by fixation with osmium tetroxide and coloration with ethyl gallate, has been applied to the respiratory system in the eggs of *Calliphora* and *Drosophila*. It has been confirmed that in eggs in their normal environment the air is confined to the inner 'spongy' layer and the vertical columns of the chorion. When *Calliphora* eggs are exposed to highly desiccating conditions the water which normally occupies the large cavities in the chorion may be replaced progressively by air. It is suggested that the function of the large air spaces in the exposed eggs of Syrphids, etc., may be water conservation.

INTRODUCTION

LEUCKART (1855), in a remarkable paper, put forward the view that the essential respiratory structure in many insect eggs is a thin porous or spongy layer which lines the shell, and which contains submicroscopic cavities filled with air, connected by means of ducts of various kinds to the outside atmosphere.

By the use of the cobalt sulphide method developed for injecting the tracheal system (WIGGLESWORTH, 1950), it was possible to demonstrate in permanent preparations the exact distribution of these air-filled layers. Most of LEUCKART's conclusions were confirmed and a number of new respiratory structures were described (WIGGLESWORTH and BEAMENT, 1950).

An improved method of tracheal injection has recently been devised, which consists in filling the system with a mixture of olive oil and kerosene in equal parts, fixing with osmium tetroxide and treating with ethyl gallate (WIGGLESWORTH, 1959b). The purpose of this communication is to illustrate the use of this method for studying the respiratory system in the insect egg.

THE EGG OF *CALLIPHORA*

A convenient object for this purpose is the egg of *Calliphora*. The structure of the egg-shell of higher Diptera was briefly described in the earlier paper (WIGGLESWORTH and BEAMENT, 1950). It is represented in Fig. 1, A. The chorion consists of a relatively thick outer layer from which tapering columns run inwards and are attached to a very thin inner layer. The columns are highly refractile and between them is a clear substance. Along the boundaries that mark the outline of

the follicular cells by which the chorion was laid down, the columns are more widely separated and they enclose conspicuous channels of the clear substance.

Running along almost the whole length of the dorsal aspect of the egg is a groove enclosed by two prominent external folds of the chorion. In the floor and walls of the furrow so produced the continuous outer layer of the chorion is interrupted and the columns, which have flattened and expanded outer ends, are freely exposed (Fig. 1, B).

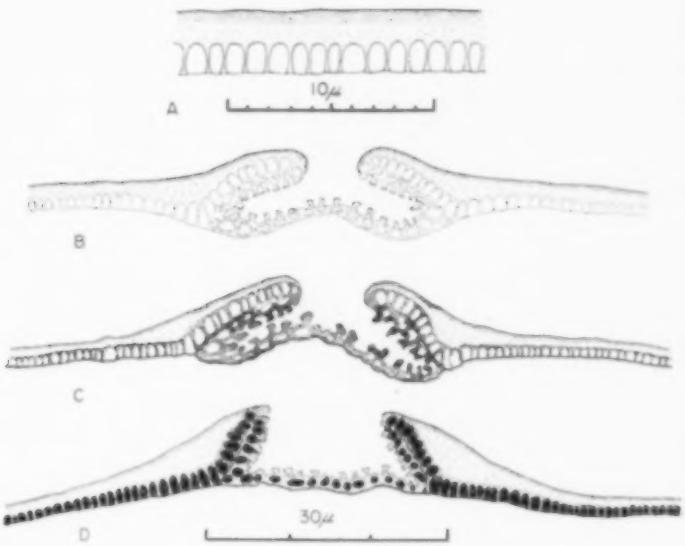


FIG. 1. (A) Chorion of *Calliphora* egg seen in transverse section. (B) Transverse section through the respiratory furrows of *Calliphora* egg: osmium tetroxide and ethyl gallate. (C) The same in egg with the air replaced by olive oil and kerosene: aqueous Bouin; osmium tetroxide; ethyl gallate. (D) The same in egg fixed with osmium tetroxide for one day before exposure to olive oil and kerosene; then followed by osmium tetroxide and ethyl gallate before section. (Severe desiccation of the egg before exposure to the oil produces the same effect.)

When the untreated egg is examined in the fresh state, the longitudinal folds appear as two silvery white lines; and a rather faint silvery film extends everywhere within the chorion. This air is readily replaced by oil if the mixture of olive oil and kerosene is brought into contact with the egg. The oil will not enter through the general outer layer of the chorion; it always spreads laterally from the longitudinal folds, which are clearly respiratory structures.

In our earlier investigations, in which we used the cobalt sulphide method of injection, we concluded that the clear substance between the columns in the chorion is water, and that in the egg of *Calliphora* under normal conditions the air is in the form of an aeroscopic sponge which composes the thin inner layer of the chorion and which extends into the vertical columns. We recognized, however,

VOL. 4 1960

that the clear spaces between the vertical columns of the egg shell in Diptera could be 'filled with liquid or with air'.

The cobalt sulphide technique is not wholly satisfactory because it is apt to form an irregular granular precipitate. The structure has therefore been reinvestigated with the osmium–ethyl gallate method. A drop of olive oil and kerosene is brought into contact with the longitudinal furrow. This is immediately cleared of air and within about half a minute the oil has spread throughout the chorion completely displacing the air. The egg is now fixed for 24 hr in Bouin's aqueous fixative, washed briefly, and placed in 2 per cent osmium tetroxide for a further 24 hr. The egg is now cut across the middle, washed in distilled water, and placed in the saturated solution of ethyl gallate for 1 day or longer. Transverse sections at 0·85–2 μ are cut by the method described elsewhere (WIGGLESWORTH, 1959a).

In Fig. 1, B is shown as a section of an uninjected egg after fixation and staining with osmium and ethyl gallate. There is only a slight darkening of the columns in the chorion; they have a pale amber colour with colourless spaces between.

In Fig. 1, C, can be seen a similar section through an egg in which the air film had been replaced by olive oil and kerosene. The inner layer of the chorion is coloured a deep blue black showing that it is permeated by the oil; the vertical columns are likewise coloured bluish and it appears to be the outermost layer of the columns which is most deeply coloured, but that may be an optical effect. The freely exposed columns in the floor and walls of the longitudinal furrow are particularly darkly coloured. There is no coloration of the spaces between the columns.

These observations confirm the results obtained with cobalt sulphide in showing that the silvery air film in the *Calliphora* egg lies in a spongy layer which forms the inner membrane of the chorion and extends also into the substance of the vertical columns.

There is one possibility for error in these observations. It might be that the oil had replaced air in the cavities between the vertical columns but that it was inaccessible to the reagents used.

In the earlier paper it was noted that if the isolated chorion was brought into contact with a mixture of kerosene and oleic acid the normal air film rapidly disappeared but within a few minutes air began to appear in the cavities between the vertical columns and very soon the water in the chorion was displaced and the entire shell was filled with air.

During the present work it was observed that if the *Calliphora* egg is placed directly into 2 per cent osmium tetroxide it acquires within a few hours a remarkably silvery appearance which persists in the chorion even when this has been removed; it is therefore not due simply to the black background produced by the osmium fixation. When eggs so treated are placed in contact with the olive oil and kerosene mixture this runs very rapidly into the shell and gives the impression that it is filling very much larger air spaces than are present in the normal chorion.

Eggs fixed with osmium tetroxide for 24 hr and then filled with olive oil and kerosene, were again fixed with osmium tetroxide and treated with ethyl gallate. Transverse sections of such eggs showed that the blue-black injection mixture completely filled the spaces between the vertical columns (Fig. 1, D).

These observations therefore confirm the conclusions of the earlier work. In the *Calliphora* egg under normal conditions the air-containing structure is the spongy 'aeroscopic' inner layer of the chorion and the air-filled sponge extends into the substance of the vertical columns. The intervening cavities are normally filled with water.

Similar observations were reported for the egg of *Drosophila*. The egg of *Drosophila melanogaster* figured in the earlier paper was still held in the ovipositor of the female at the time of injection, but the normal air-containing layer was already established. The results have now been confirmed on *Drosophila* eggs some hours after laying on moist filter paper. In these eggs also the replacement of the air in the shell by olive oil and kerosene gave no evidence that this air is in the spaces between the vertical columns.

VOL. 4 1960

As previously described (WIGGLESWORTH and BEAMENT, 1950) the *Drosophila* egg when laid unattached on moist filter paper will float in water with the horns in the surface film and the egg submerged. This observation served to illustrate very clearly the respiratory function of the horns on this egg (which did not appear to have been recognized previously in the case of the *Drosophila* egg). But as HINTON (1959) points out, the egg is heavier than water and, as with the 'floats' of *Anopheles* larvae, for example, the egg is supported by surface forces and not by buoyancy. There is no suggestion that these horns function as 'floats' in nature.

DISCUSSION

In the eggs of Syrphids laid exposed on leaves (WIGGLESWORTH and BEAMENT, 1950) and probably in the exposed eggs of Tachinids (PANTEL, 1913), the chorion is very much thicker than that of *Calliphora* and the cavities between the columns are always filled with air. There is no reason to suppose that the oxygen required by the *Calliphora* egg is less than that required by Syrphids and Tachinids. Why then does this egg not have the large cavities of the chorion normally filled with air? It seems likely that, as in most other insect eggs, the essential respiratory structure is the air-filled spongy protein of the shell substance, and the air in the large cavities is not there primarily for respiratory purposes. Perhaps these air-filled spaces are to be thought of rather as having a water-conserving function. A layer of air between two cuticles forms a highly efficient mechanism for reducing transpiration—as in the puparium of *Calliphora* (WIGGLESWORTH, 1945; BEAMENT, 1959).

In a recent report HINTON (1959) claims that the air in these eggs of Diptera, notably in *Drosophila*, is not normally confined to an 'aeroscopic sponge' (as described above), but fills the cavities between the vertical columns, as it does in Syrphid eggs. The refractive effects produced by air are so disturbing that it is almost impossible to determine its location by simple observation under the

microscope. HINTON, however, confirmed his conclusions by 'heavy injection' with cobalt naphthenate.

If the egg is simply brought into contact with oil, this runs into the shell and replaces the air completely. No more oil will enter after 'heavy injection'. That suggests that perhaps the prolonged exposure to a vacuum in Hinton's experiments led to the evaporation of the water in the shell and its replacement by air. Alternatively, we have seen that prolonged contact of the chorion with certain oils may lead to the replacement of the water by air and this in turn may then be replaced by oil.

Eggs of *Calliphora* at varying stages of development were removed from the meat on which they had been laid and were divided into two lots. One lot was kept on dry filter paper over calcium chloride at room temperature for 4 hr; the other lot was kept in a humid atmosphere over damp filter paper for the same length of time. The air in the chorion of both lots was then replaced by olive oil and kerosene and transverse sections prepared as before. In the eggs maintained in a moist atmosphere the air in the shell was again confined to the inner layer of the chorion and the substance of the columns. In the eggs exposed to desiccating conditions air had extended for a variable distance into the space between the vertical columns. In some specimens this space was completely filled with the injection mixture in the neighbourhood of the longitudinal furrow. Further out it was limited to the channels along the cell boundaries. And further out still it was confined to the spongy substance of the chorion.

A somewhat similar situation exists in the egg of *Dixippus* as previously described (WIGGLESWORTH and BEAMENT, 1950). The essential aeroscopic membrane is the innermost layer of the chorion. Outside this lies a system of vertical pillars or vertical cones (themselves containing an air sponge) with water between. As embryonic development proceeds the water slowly evaporates and free air spreads into this layer from the respiratory pore. This air can be seen by trans-illumination in the living egg and the presence of water at the periphery of the 'air bubble' is clearly indicated by the smooth sharp boundary between air and liquid.

The essential problem of the air-filled sponge remains: the mechanism by which the fluid content is replaced by air. In the earlier paper this was attributed to tanning, with or without wax secretion, leading to the development of hydrofuge properties in the spongy substance. It is interesting that fixation with osmium tetroxide apparently has a similar effect on the water-filled cavities of the chorion. The possible physico-chemical nature of this change will be discussed elsewhere (BEAMENT, 1960).

REFERENCES

BEAMENT J. W. L. (1959) The waterproofing mechanism of arthropods—I. The effect of temperature on cuticle permeability in terrestrial insects and ticks. *J. exp. Biol.* **36**, 391–422.

BEAMENT J. W. L. (1960) The wetting properties of insect cuticle. *Nature, Lond.* In press.

HINTON H. E. (1959) Plastron respiration in the eggs of *Drosophila* and other flies. *Nature, Lond.* **184**, 280–281.

LEUCKART R. (1855) Über die Micropyle und den feinern Bau der Schalenhaut bei den Insekteneiern. *Arch. Anat. Physiol., Lpz.* **1855**, 90–264.

PANTEL J. (1913) Recherches sur les diptères à larves entomobies—II. Les enveloppes de l'oeuf avec leur dépendances, les dégâts indirects du parasitisme. *Cellule* **29**, 1–289.

WIGGLESWORTH V. B. (1945) Transpiration through the cuticle of insects. *J. exp. Biol.* **21**, 97–114.

WIGGLESWORTH V. B. (1950) A new method for injecting the tracheae and tracheoles of insects. *Quart. J. micr. Sci.* **91**, 217–224.

WIGGLESWORTH V. B. (1959a) A simple method for cutting sections in the 0·5–1 μ range, and for sections of chitin. *Quart. J. micr. Sci.* **100**, 315–320.

WIGGLESWORTH V. B. (1959b) The role of the epidermal cells in the 'migration' of tracheoles in *Rhodnius prolixus* (Hemiptera). *J. exp. Biol.* **36**, 632–640.

WIGGLESWORTH V. B. and BEAMENT, J. W. L. (1950) The respiratory mechanisms of some insects eggs. *Quart. J. micr. Sci.* **91**, 429–452.

VOL.
4
1960

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

G. BECHT, G. HOYLE, and P. N. R. USHERWOOD: Neuromuscular transmission in the coxal muscles of the cockroach

L. HUOT and W. VERLY: Dosage du ^{32}P total d'une larve vivante de *Tenebrio molitor* L. (Coléoptère, Tenebrionidae)

H. D. PARK and J. BUCK: The relation of oxygen consumption to ambient oxygen concentration during metamorphosis of the blowfly, *Phormia regina*

S. D. BECK and W. HANEC: Diapause in the European corn borer, *Pyrausta nubilalis* (Hübn.)

P. W. MILES: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera, Lygaeidae)—II. Physical and chemical properties

G. F. HOWDEN and B. A. KILBY: Biochemical studies on insect haemolymph—II. Variations in reducing power with age and the effect of diet

M. B. SHYAMALA, K. SHARADA, M. G. BHAT, and J. V. BHAT: Chloromycetin in the nutrition of the silkworm, *Bombyx mori* L.—II. Influence on digestion and utilization of protein, fat, and minerals

H. LANGER: Die Wirkung von Licht auf den chemischen Grundaufbau des Auges von *Calliphora erythrocephala* Meig.

B. J. SELMAN: On the tissue isolated in some of the larval appendages of *Sialis lutaria* L. at the larval–pupal moult

R. W. NEWBURGH, L. N. POTTER, and V. H. CHELDELIN: Oxidative phosphorylation in *Phormia regina* larvae

P. W. MILES: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* Dall. (Heteroptera, Lygaeidae)—III. Origins in the salivary glands

R. H. DADD: The nutritional requirements of locusts—I. Development of synthetic diets and lipid requirements

K. U. CLARKE: Studies on the relationships between air temperature and the internal body temperature of *Locusta migratoria*

H. CHINO: Enzymatic pathways in the formation of sorbitol and glycerol in the diapausing egg of the silkworm, *Bombyx mori*—I. On the polyol dehydrogenases

L. Y. FINLAYSON: A comparative study of the effects of denervation on the abdominal muscles of saturniid moths during pupation

R. H. HACKMAN and M. GOLDBERG: Composition of the oothecae of three Orthoptera

D. S. ANDERSON: The respiratory system of the egg-shell of *Calliphora erythrocephala*

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 191 *to* 201. *Pergamon Press Ltd., London. Printed in Great Britain*

NEUROMUSCULAR TRANSMISSION IN THE COXAL MUSCLES OF THE COCKROACH

G. BECHT,* G. HOYLE, and P. N. R. USHERWOOD

Department of Zoology, University of Glasgow

(*Received* 5 *June* 1959)

Abstract—The coxal muscles of the cockroach are considered to be of two kinds, 'slow' and 'fast', according to their mechanical responses. In the present investigations the electrical responses of the two kinds of muscle were examined with intracellular electrodes. It is evident from these responses that the coxal muscles are each innervated by typical 'fast' axons giving evoked action potentials from the muscles which are of a uniform type. The 'slow' muscles receive in addition a 'slow' axon which gives rise only to facilitating junctional potentials. The mechanical differences are due to intrinsic properties of the muscle fibres, not to differences in neuromuscular transmission mechanisms at the synaptic level.

VOL.
4
1960

INTRODUCTION

THE adjustment of tension, length, and speed of contraction in the skeletal muscles of insects is accomplished in ways which differ considerably from those found in vertebrates. Only in a few muscles, mainly flexors, is the contraction adjusted in part by variation in the number of motor units active, and then only in a limited way, since the number of such units is very small (HOYLE, 1957). Many muscles of insects consist of a single unit, sometimes supplied by only a single axon (e.g. tymbal muscle of cicada, PRINGLE, 1954), but commonly there are two motor axons supplying each unit (PRINGLE, 1939; HOYLE, 1955a; WOOD, 1958). One of these axons evokes a brisk twitch response, whereas the other evokes only a minute response or no response to a single shock. The former type of axon is referred to as a 'fast' axon, the latter as a 'slow' one.

The electrical responses to the 'fast' axon are all-or-nothing events showing no facilitation. They take the form of large post-synaptic (junctional) potentials which evoke varying degrees of electric response from the muscle fibre membrane. The latter are graded events depending on the electrical excitability of the membrane; their nature (in another orthopteran insect), has been discussed by CERF *et al.* (1959). The 'slow' axon gives rise to electrical responses not in every muscle fibre but only in a proportion. In a small, weak muscle the proportion is high (HOYLE, 1959), in a powerful one it is low (HOYLE, 1955b).

The muscle fibres which show the 'slow' responses also show 'fast' ones, with rare exceptions. In the jumping muscle of the locust two kinds of 'slow' response were distinguished (HOYLE, 1955b), but in other muscles of the same animal only

* Department of Zoology, University of Nijmegen, Holland.

one of these two kinds is commonly encountered. This consists of a junctional potential, the height of which varies greatly from fibre to fibre. The larger ones give rise to small electrogenic responses. The 'slow' axon responses are mainly characterized by the extent to which they show facilitation during repetitive excitation starting after a period of rest. This is conspicuous when the initial response is small. The mechanical responses increase greatly in rate and extent of shortening, and in tension development, with increasing frequency of excitation.

Following the above pattern, contraction in a doubly-innervated insect muscle is regulated principally by the frequency of discharge of its 'slow' axon, with brief discharges from the 'fast' axon coming in to give especially powerful or quick contractions when needed, particularly for escape reactions.

In contrast with this picture Dresden (1956), Becht and Dresden (1956), and Becht (1959) have found that in the coxal muscles of the cockroach the contractions of different muscles are markedly different even when the motor nerves are maximally stimulated. Muscles 136 and 137, for example, give a large twitch in response to a single shock so that there is little further increase in extent of shortening of the muscle under tetanus. Muscle 135b gives only a weak twitch and extends the trochanter fully only during stimulation at a high frequency. The former muscles are innervated by only a single axon, which they share (Dresden and Nijenhuis, 1958), the latter (135b) is innervated by three or four axons of different diameters. The muscles differ slightly in colour, 136 and 137 being white and 135b pinkish. Becht (1959) has called these muscles 'fast' and 'slow' respectively. In the locust *Locusta migratoria* Ewer (1957) found that the main thoracic muscles could be divided into two groups according to their reaction to directly-applied electrical stimulation. Stimulation of this kind is thought to act indirectly in insects via the motor nerve. In one group, the muscles responded to single shocks with vigorous twitch contractions. In the other group they responded with an appreciable contraction only when stimulated repetitively at a moderate frequency. Ewer referred to the two kinds as twitch and tonic respectively, but they may well correspond to Becht's 'fast' and 'slow' categories. Other muscles of the cockroach thorax and coxa fall into one or other of the two categories but for a few exceptions, which can be classified as intermediate between the two.

Some of the cockroach 'fast' and 'slow' muscles have been studied in transverse section by Smit (1958). He found differences in histological appearance, the 'fast' muscles being composed wholly or in part of irregularly-shaped fibres having a hollow core with the fibrils arranged in radial lamellae. The slow ones had a high proportion of rounded fibres of larger diameter tightly packed with small fibrils, uniformly distributed. Both kinds of histological picture have been seen frequently in different insect muscles (Tiegs, 1955) but they had not previously been associated with physiological differences. The appearances could be fixation artefacts, but as Smit points out, their constancy nevertheless demonstrates some kind of real difference in reaction to fixatives, which must in turn reflect physiological differences. More study is needed on this important point.

On the evidence available to them from stimulation maximally of the whole nerve-muscle system, BECHT and DRESDEN (1956) and EWER (1957) could not rule out the possibility that the differences they observed in mechanical reaction of different muscles might have been due to differences in the physiology of neuromuscular transmission to the muscles they examined. They did not consider the possibility of 'slow' axons being responsible for slow contractions, nor did DRESDEN and NIJENHUIS (1958). Instead, their interpretations were based on assumed differences in contractile properties of the muscle fibres. WILSON (1954) had earlier claimed there are two distinct kinds of muscle fibre in the cockroach, which he also referred to as 'fast' and 'slow'. The conclusions of WILSON have, however, been severely criticized by HOYLE (1957) on the grounds that the electrical results on which he based his conclusions could be interpreted as the differences between recordings from fibres in good, as compared with poor, condition. He did not selectively stimulate the slow axons already found by PRINGLE (1939) but nevertheless claimed that slow contractions in the animal are the result of excitation of 'slow' muscle fibres. In order to clear up these conflicting interpretations and to determine the extent to which there are differences in neuromuscular transmission mechanisms between the different muscles, experiments are urgently required which will correlate electrical with mechanical events during separate stimulation of all the motor axons supplying muscles of the different types claimed. In the present experiments we have studied with the aid of intracellular recording electrodes the responses of those muscles located in the coxa of the cockroach which had been earlier studied mechanically by BECHT and DRESDEN (1956) and BECHT (1959).

VOL. 4 1960

METHODS

The mesothoracic legs of large, mature male cockroaches (*Periplaneta americana*) in nearly-intact animals were used. The muscles were prepared according to the methods of BECHT (1959) and designated according to the scheme of CARBONELL (1947). The muscles investigated were numbers 135b, 136, and 137 (extensors); 138, 139a, b, and c, and 140 (flexors).

The animals were lightly anaesthetized with carbon dioxide and partly embedded on their backs in plasticine. The abdomens were left as free as possible and in contact with air either throughout the experiment or up to the last minute before recording. The mesothoracic ganglion was exposed, and the majority of nerves, except those relevant for motor discharge to the muscle under examination and the corresponding reflex sensory nerves, were severed so as to reduce movements to a minimum. Paired, hooked silver electrodes were then placed under the appropriate motor nerve (DRESDEN and NIJENHUIS, 1958) and the coxa adjusted to permit exposure of the muscle. The overlying cuticle and hypodermis were next dissected away. To expose muscle 135b it was necessary to dissect away adjacent muscles. The other muscles examined could be observed without further dissection. Where possible the muscle was examined from both its ventral and also (after adjustment of the coxa) its dorsal surface (or internal/external, according to the position of the coxa).

The exposed part was surrounded by a wall of plasticine and immersed in a Ringer's solution having the following composition: 9·32 g NaCl, 0·77 g KCl, 0·50 g $CaCl_2$, 0·18 g $NaHCO_3$, 0·01 g NaH_2PO_4 to 1 l. of distilled water. Stimulalation was by rectangular pulses controlled as to strength and duration, and recording was with the aid of 3 M KCl-filled glass capillary micro-electrodes with conventional amplifier and display circuitry.

RESULTS

A special search has been made in each muscle for the presence of electrical potentials of the type designated S_{1b} in the locust by HOYLE (1955b), i.e. for small synaptic potentials showing facilitation during repetition, which are characteristic responses to 'slow' axons in arthropods. This required recording from several muscle fibres in each muscle during the evocation of reflex contractions. In some preparations the latter were facilitated by first treating the animal with γ-benzene hexachloride (10^{-6}), although this method was not always successful. The ganglion has also been painted with nicotine in order to cause the motor neurons to discharge. This method has revealed the presence of 'slow' axons supplying some of the muscles, but it is laborious and wasteful of cockroaches because there is only a very brief discharge followed by blocking. During the discharge there is time to record from only one muscle fibre, and only a small proportion of the muscle fibres does in fact respond to 'slow' axons.

EXTENSORS

Muscles 136 and 137. These two muscles are anatomically distinct, being situated on opposite sides of the coxa. But functionally, they act as a single unit, causing extension of the trochanter (BECHT and DRESDEN, 1956). They are supplied by a single, shared axon (NIJENHUIS and DRESDEN, 1958) (confirmed in our experiments). We have not found any evidence for the presence in the muscle fibres of electrical responses of more than one kind.

The resting potentials obtained in different muscle fibres varied from 40 to almost 70 mV. The electrical responses were all similar, apart from small variations, no matter from which muscle fibre or part of a muscle fibre they were recorded. Some examples are given in Fig. 1. In some records there is a slight inflexion of the rising phase at a value of about 20 mV inside negative with respect to zero. This point of inflexion denotes the very slight delay between the peak of the rising phase of the junctional potential and the resulting electrically excited response. These potential changes are orthodox action potentials of the 'fast' kind, which have been described previously in the extensor tibialis muscle of the same species (HOYLE, 1955c). An overshoot (reversed membrane potential) is present in many fibres, reaching as much as 20 mV inside positive.

Spontaneously-occurring, reflex-evoked or nicotine-induced potentials were all of this same kind. It was noticed, however, that whenever the responses were at all closely spaced the second and subsequent responses were rather small. This is due to refractoriness of a very prolonged kind (Fig. 1 f). Both the post-synaptic

potentials and the active membrane responses are markedly relatively refractory, the former for approximately 15 msec, the latter for up to 100 msec. Even a large junctional potential fails to evoke a secondary response of more than a very few millivolts if it arrives within 50 msec following the elicitation of a large, overshooting spike.

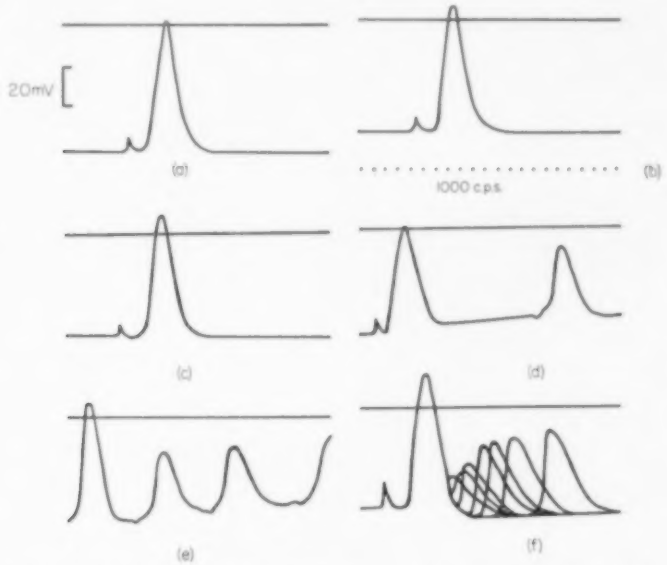


FIG. 1. Electrical responses to motor nerve stimulation recorded from different single muscle fibres of the extensor trochanter motor unit, muscles 136 and 137, recorded with an intracellular electrode. (a) 136; (b–f) 137. The second response in (d) and all responses in (e) occurred naturally due to reflex or spontaneous action. Note the marked refractoriness of the responses. The different relative refractory periods of the junctional potential and of the active membrane response are evident as the motor nerve was stimulated repeatedly with a pair of shocks whilst gradually increasing the interval between the shocks. The upper line in each record indicates zero membrane potential. Temperature 10–20°C.

The mechanical response fatigues very quickly under tetanus (BECHT and DRESDEN, 1956). This is associated with a very rapid decline in the height of the electrical responses and is presumably attributable to a failure of synaptic transmission, due perhaps to depletion of transmitter substance.

Muscle 135b. This muscle is a particularly interesting one because it is the 'slowest' muscle encountered by BECHT and DRESDEN. According to DRESDEN and NIJENHUIS (1958) it is innervated by one large, one medium-sized, and possibly two small-sized axons. In our experiments we have found three axons, as evidenced by the electrical responses. Two of these are 'fast' axons innervating two separate bundles of fibres with perhaps a very small degree of overlap of their fields. The two axons had different thresholds and could be separately stimulated. The corresponding mechanical responses appeared to be quite vigorous twitches under

VOL. 4 1960

the microscope. BECHT and DRESDEN (1956) also noticed this, although they found it hard to record a mechanogram owing to the weakness of the contraction. The electrical responses recorded from the two muscle units did not differ appreciably.

The innermost bundle of muscle fibres gave also some responses which fulfilled the requirements of a 'slow' axon, i.e. they were facilitating junctional potentials. They were located in a little more than one-third of the muscle fibres, so that the extent of double-innervation is probably of the order of 40 per cent. Typical responses are shown in Fig. 2. On no occasion was any evidence obtained that the other bundle is also innervated by a 'slow' axon.

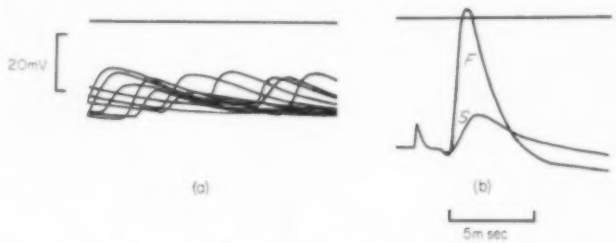


FIG. 2. Electrical responses of a muscle fibre of muscle 135b. (a) Reflexly-evoked 'slow' axon responses. (b) Responses to separate stimulation of the 'slow' axon (*S*) and the 'fast' axon (*F*). Critical width of square pulse was used to separate the more slowly-conducted impulse of the 'slow' axon from that of the 'fast'. Temperature 19–20°C.

BECHT and DRESDEN found that following an increase in the voltage of stimulation applied to the nerve (nerve 4, ramus 2 b) there was sometimes a sharp decrease of the contraction height. They suggested that, by analogy with comparable results in some crustacean preparations, this might denote the presence of an inhibitory axon. We have obtained no evidence for such an axon in the present experiments. If the motor axons may become blocked due to anodal polarization at the stimulating electrodes when the stimulus strength is raised, that could give a sudden relaxation during continued stimulation. In any case, it is extremely unlikely that the response to stimulation of the nerve maximally could, even in principle, be inhibited by an inhibitory axon. The response to stimulation of a 'fast' axon, if this gives rise to a spike-like electrical response, cannot be inhibited by the inhibitory axons in Crustacea; and the electrical response in the insect muscle concerned is even larger than the occasional spike-like response of crustaceans.

The electrical response to the 'fast' axon fatigues just as rapidly as that of muscles 136 and 137. Thus the mechanogram obtained by stimulating the nerve maximally at high frequency (BECHT and DRESDEN, Fig. 4) could be due as much to the response to the 'slow' axon, which does not fatigue, as to the 'fast'. The contraction steps following stepwise increase in stimulation frequency closely resemble those given by the locust muscles when the 'slow' axon alone is stimulated, and, like the latter, the responses do not show any sign of fatigue over several minutes.

Nevertheless, summative responses of contractions in response to repetitive excitation of the 'fast' axon probably also contribute initially to the development of tension.

135b is an extensor and quite the weakest of the whole group. 136 and 137 are innervated only by a 'fast' axon giving strong twitches. 135a and c also give large twitch responses (as measured by shortening against a light isotonic lever). Thus it may be that tonic extension at the coxo-trochanteral joint is undertaken by the 'slow' axon supplying 135b, and moderately slow movements by the two 'fast' axons which innervate it. Faster walking and running movements would be served by 135a and 135c together with 136 and 137.

FLEXORS

Muscle 138. This muscle is a flexor of the trochanter, innervated according to DRESDEN and NIJENHUIS (1958) from nerve 3b. However, following section of 3b

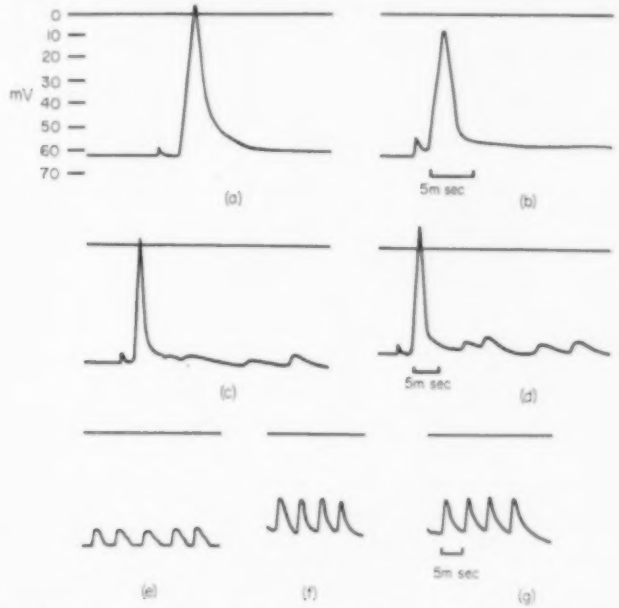


FIG. 3. Electrical responses of muscle fibres of muscle 138. (a) and (b) Two typical examples of 'fast' axon response. (c) and (d) Two examples of responses to 'fast' axons recorded on a slower time-base than (a) and (b), showing spontaneously-occurring responses to discharge in the 'slow' axon occurring in the same fibres as the evoked responses to the 'fast' axon. (e–g) Successive records from the same muscle fibre obtained during a discharge of the 'slow' axon evoked by coating the ganglion with nicotine. Note the 'facilitation'. Temperature 19–20°C.

we have obtained reflex responses from part of this muscle, although we have not located their source. BECHT and DRESDEN (1956) regarded the mechanical responses of this muscle as being of intermediate type; it gives twitches but these are small

VOL. 4 1960

and there is appreciable summation of mechanical responses even at a frequency as low as 1/sec.

When the nerve 3b is maximally stimulated, electrical responses which resemble the 'fast' axon ones of other muscles appear in every muscle fibre. They also show very long refractory periods. In addition there is a 'slow' axon. Responses to this axon are readily observed because they are present in about 50 per cent of the muscle fibres. They are of different heights in different muscle fibres. The smaller ones show considerable facilitation during repetition (Fig. 3 c–g).

Thus the mechanogram obtained by BECHT and DRESDEN must have been a compound one, giving the summated responses to stimulation of the two axons, 'fast' and 'slow', simultaneously.

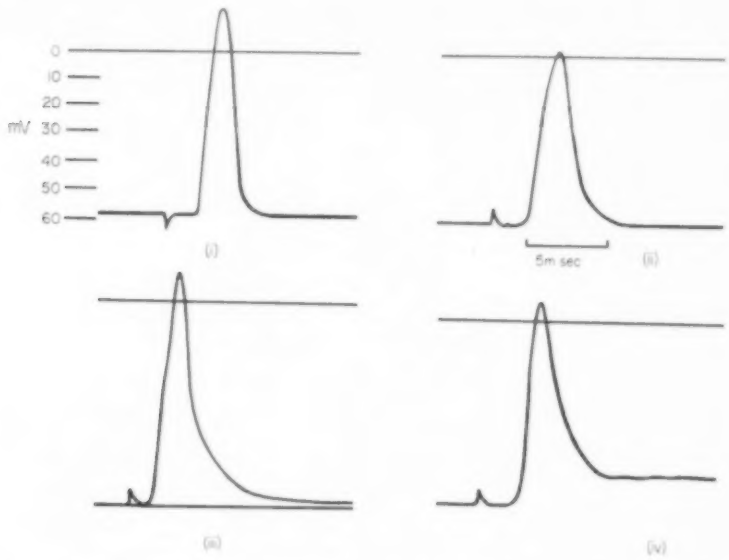


FIG. 4. Examples of electrical responses, all of the kind due to 'fast' axon discharge, obtained during stimulation of the motor nerves supplying different flexor muscles of the coxa, numbers 140 (i), 139a (ii), and 139b (iii) and (iv). Note the inflexion in the rising phase in (iii) (muscle 139b), the largest response obtained in these investigations. Temperature 19–20°C.

Muscle 139c. This is a powerful, thick flexor muscle lying next to 138 and innervated from nerve 3b. DRESDEN and NIJENHUIS found at least five axons supplying this muscle. Like other flexor muscles (HOYLE, 1957) it is of multiple-unit type. There are two separate parts at least, each with its own 'fast' axon. We have also seen reflex responses which indicated the presence of at least one 'slow' axon, but only encountered the typical 'slow' axon electrical responses in two muscle fibres, out of about forty penetrated, when the muscle was obviously giving (by direct visual observation) a slow contraction. The percentage innervation

must be very small, possibly of the order of 10 per cent, as in the powerful locust jumping muscle (HOYLE, 1955b).

Muscles 139a and 139b. These muscles each contain two or three units, as evidenced by the fact that different regions respond as the stimulus strength is very gradually increased. We have obtained only responses of the kind given by 'fast' axons, from these muscles. An example is given in Fig. 4.

Muscle 140. This muscle receives, according to NIJENHUIS and DRESDEN (1958), three motor axons. We have examined the muscle extensively, and have found evidence for two or three axons, but all these axons are of the 'fast' kind, innervating different muscle bundles. There is no evidence of a 'slow' innervation. Thus most of the flexors have no, or a very sparse, 'slow' axon supply.

DISCUSSION

The results of the present experiments make it quite clear that the neuromuscular mechanisms present in the cockroach coxal muscles are essentially similar to those obtaining in the ordinary skeletal muscles of locusts (HOYLE, 1955b) and the stick insect *Carausius* (WOOD, 1958). Every single muscle fibre of each muscle receives an innervation from an axon of the 'fast' type which elicits a rather uniform kind of electrical response which has been analysed previously (HOYLE, 1955c). In some muscles no other innervation is present; some of the flexor muscles receive more than one of these 'fast' axons. The different axons then innervate separate bundles of muscle fibres, so that the whole has a small number of motor units.

In addition to the 'fast' axon or axons, some of the muscles receive one or more 'slow' axons. These give responses which are junctional potentials of various magnitudes. They show extensive growth by facilitation during repetition. The occurrence of the slow and fast responses in the same muscle fibre makes it quite certain that these muscle fibres receive a double innervation.

The principal question which now confronts us is whether it is justifiable to refer to the different muscles as 'slow' or 'fast' muscles as did BECHT and DRESDEN (1956). The present experiments have ruled out the possibility that the different responses can be attributed to differences in the physiology of neuromuscular transmission to the different muscles. In this respect the opinion of BECHT and DRESDEN, and BECHT (1959), is strengthened. On the other hand, the muscles which showed the 'slow' kind of contraction have been shown by us to receive an innervation from a 'slow' axon. The muscles which gave the best 'fast' response were also those which are not supplied by a 'slow' axon.

Since BECHT and DRESDEN stimulated the whole nerves the possibility cannot be ruled out without further experiment that their results could be attributed to this difference in innervation.

It may, however, be that the presence of 'slow' axons with their different synaptic reactions comes to be associated with physiological differences in the muscle fibres which they innervate. If this were the case it would, perhaps, be possible to distinguish two kinds of muscle fibre on this basis.

VOL. 4 1960

The alkaloid ryanodine causes a rapid block of some muscles, a much slower one in others, which continue to respond, although sluggishly, for a long time (BECHT and DRESDEN, 1956). It is precisely the former muscles which have only a 'fast' axon innervation and the latter which also have a 'slow' one. This affords us a very important clue for further investigations on the pharmacology of muscular activity in insects, about which almost nothing is known.

The main effect of the present results is to refer the problem of differences in kind of contraction to possible intrinsic ones in the muscle fibres. But it is unfortunate that it cannot yet be considered proven that there are real differences in contraction. The results of BECHT and DRESDEN were obtained by using a simple lever attached to the segment on which the appropriate muscle acts and recording its movements on a horizontal kymograph. Those of EWER were simply based on visual inspection. It will be necessary to investigate the same muscles critically, if this can be achieved physically, in regard to development of tension under isometric conditions, and speed and extent of shortening under various loads, before the reality of the differences, and their qualities, become established. Also, these experiments will have to be done whilst stimulating only the 'fast' axons if they are to provide truly comparable results.

SUMMARY

(1) The electrical responses to nervous stimulation of several different muscles of the coxa of the cockroach, *Periplaneta americana*, have been examined with the aid of intracellular recording micro-electrodes.

(2) All the muscle fibres were found to be innervated by one or more 'fast' axons giving rise to a large, all-or-nothing, often overshooting electrical response in each muscle fibre. These responses were substantially similar in all the muscles investigated.

(3) Some of the muscles, notably 135b and 138, were found to receive a 'slow' axon in addition to the 'fast' ones. Only a fraction of the muscle fibres were dually responsive, the majority showing no response to the 'slow' axon. The responses to the 'slow' axon consisted largely of junctional potentials of various heights. The smaller ones showed a conspicuous growth (facilitation) during repetition.

(4) The results were similar to those found previously in skeletal muscles of locusts.

(5) No evidence was found which would justify a division of the cockroach muscles into 'slow' and 'fast' muscles on the basis of their neuromuscular electrical responses. Any differences there may be in mechanical responsiveness must reside in the contractile properties of the muscle fibres themselves, or in the mechanism coupling excitation to contraction.

REFERENCES

BECHT G. (1959) Studies on insect muscles. *Bijdr. Dierk.* **29**, 1–36.

BECHT G. and DRESDEN D. (1956) Physiology of the locomotory muscles in the cockroach. *Nature, Lond.* **177**, 836.

CARBONELL C. S. (1947) The thoracic muscles of the cockroach *Periplaneta americana* L. *Smithson. misc. Coll.* **107**, 1–23.

CERF J. A., GRUNDFEST G., HOYLE G., and MCCANN F. V. (1959) The mechanism of dual responsiveness in muscle fibres of the grasshopper *Romalea microptera*. *J. gen. Physiol.* **43**, 377–396.

DRESDEN D. (1956) A technique for recording muscle contractions in an insect on a kymograph. *Nature, Lond.* **177**, 835.

DRESDEN D. and NIJENHUIS E. D. (1958) Fibre analysis of the nerves of the second thoracic leg in *Periplaneta americana*. *Proc. Acad. Sci. Amst.* **61**, 213–223.

EWER D. W. (1957) Notes on acridid anatomy—III. Twitch and tonic muscles in the thorax of Locusta. *J. ent. Soc. S. Afr.* **20**, 231–233.

HOYLE G. (1955a) The anatomy and innervation of locust skeletal muscle. *Proc. roy. Soc.* (B) **143**, 281–292.

HOYLE G. (1955b) Neuromuscular mechanisms of a locust skeletal muscle. *Proc. roy. Soc.* (B) **143**, 346–367.

HOYLE G. (1955c) The effects of some common cations on neuromuscular transmission in insects. *J. Physiol.* **127**, 90–103.

HOYLE G. (1957) The nervous control of insect muscle. In *Recent Advances in Invertebrate Physiology* (Edited by Scheer B. T.). University of Oregon Press.

HOYLE G. (1959) Neuromuscular mechanisms of an insect spiracular muscle. *J. Ins. Physiol.* **3**, 378–394.

PRINGLE J. W. S. (1939) The motor mechanism of the insect leg. *J. exp. Biol.* **16**, 220–231.

PRINGLE J. W. S. (1954) The mechanism of the myogenic rhythm of certain insect striated muscles. *J. Physiol.* **124**, 269–291.

SMIT W. A. (1958) Muscle fibres of the fast and slow contracting muscles of the meso-thoracic leg of the American cockroach (*Periplaneta americana* L.). *Nature, Lond.* **181**, 1073–1074.

TIEGS O. W. (1955) The flight muscles of insects—their anatomy and histology: with some observations on the structure of striated muscle in general. *Phil. Trans.* (B) **238**, 221–348.

WILSON V. J. (1954) Slow and fast responses in cockroach muscle. *J. exp. Biol.* **31**, 280–290.

WOOD D. W. (1958) The electrical and mechanical responses of the prothoracic flexor tibialis muscle of the stick insect *Carausius morosus* Br. *J. exp. Biol.* **35**, 850–861.

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 202 *to* 208. *Pergamon Press Ltd., London. Printed in Great Britain*

DOSAGE DU ^{32}P TOTAL D'UNE LARVE VIVANTE DE *TENEBRIO MOLITOR* L. (COLÉOPTÈRE : TENEBRIONIDAE)

L. HUOT* et W. G. VERLY†

Institut Léon Fredericq, Biochimie (Laboratoire des Isotopes), Université de Liège et Centre Belge de l'Energie Nucléaire

(*Received* 1 *August* 1959)

Abstract—It is possible to determine total ^{32}P in living larvae of *Tenebrio molitor* with an error of ± 5 per cent, which is related specially to the error in the correction factor utilized. The very small variation of this correction factor in relation to the weight of larvae is discussed.

INTRODUCTION

Le problème du dosage du ^{32}P total d'un *Tenebrio molitor* vivant n'est pas insoluble puisque le diamètre d'une larve de cet insecte ne dépasse pas 3,5 mm tandis que la portée maximum du rayonnement β du ^{32}P dans un milieu de densité unitaire est de 7 à 8 mm. Il faut néanmoins prévoir une correction pour l'absorption d'une partie du rayonnement dans les tissus de l'insecte; la valeur de ce coefficient de correction en fonction du poids de la larve est établie expérimentalement dans le présent travail.

METHODES ET RESULTATS

Nous avons utilisé des *Tenebrio molitor* de races F et G (Leclercq, 1950) qui proviennent de nos élevages réguliers maintenus à 27°C, à une humidité relative d'environ 70 pour cent, et nourris de farine non blutée.

Pour la préparation de la nourriture radio-active, nous tamisons fortement la farine à laquelle nous ajoutons 5 pour cent de levure desséchée pour parer aux déficiences vitaminiques. Pour 100 g de ce mélange, nous ajoutons 100 μc de phosphate marqué. Après avoir broyé à l'aide d'un mortier et mélangé quelques heures dans un moulin à billes, il est permis d'émettre l'hypothèse que, à l'echelle de la bouchée du ver, le phosphore de la farine a partout la même radio-activité spécifique. Les larves sont placées dans des récipients qui contiennent ce mélange radioactif puis nous les rangeons dans une étuve où les conditions sont les mêmes que pour nos élevages réguliers.

Les mesures de radio-activité sont faites avec un tube de Geiger Tracerlab TGC-2. Tous les résultats sont corrigés pour le mouvement propre du compteur et la décroissance de la radio-activité. Pour cette dernière correction, nous utilisons

* Titulaire d'une Bourse postdoctorale du Conseil National de Recherche du Canada et boursier du Conseil des Recherches Agricoles de la province de Québec.

† Associé du Fonds National Belge de la Recherche Scientifique.

une source de ^{32}P préparée à partir de la solution qui sert à marquer la farine et recouverte d'une pellicule d'un micron environ de plastique.* Ce recouvrement ne modifie aucunement le nombre de désintégrations enregistrées par le compteur comme le montre le Tableau 1.

TABLEAU 1—MESURES SUR TROIS ETALONS DE ^{32}P AVANT ET APRÈS LE RECOUVREMENT DE PLASTIQUE

Etalons	c/5 min		Différence	't'
	Avant le recouvrement	Après le recouvrement		
1	10 071 ± 100	10 185 ± 101	+114	0,80
2	9895 ± 99	9983 ± 100	+88	0,62
3	10 417 ± 102	10 219 ± 101	−198	1,37

La mesure de la radio-activité faite sur la larve entière doit être parfaitement reproductible. Dans ce but, un petit cylindre en duralumin a été creusé d'une gouttière en couronne (Fig. 1) dont le centre vient se placer dans l'axe du tube

VOL. 4 1960

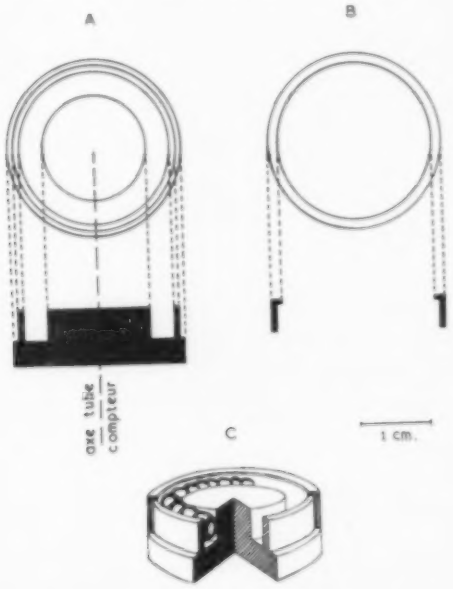


FIG. 1. Petite cage pour mesurer le ^{32}P total d'une larve vivante de *Tenebrio molitor*. A—Plan et coupe de la cage. B—Plan et coupe de l'anneau qui tient la feuille de cellophane. C—Vue de la larve dans la cage.

* Dissoudre 10 mg de plexiglass dans 10 ml de chloroforme. Placer 0,5 ml de cette solution sur le disque métallique qui contient le ^{32}P et laisser évaporer.

compteur au moment de la mesure. De cette manière, la larve en se déplaçant dans la gouttière ne modifie pas sa géométrie par rapport au compteur. La cage est fermée par une feuille de cellophane tenue par un anneau. Au cours de toutes nos séries de mesures, nous n'avons pas observé de contamination de cette cage. Dans ces conditions, les mesures faites sur une même larve ne montrent pas une variabilité qui dépasse l'erreur statistique (égale à la racine carrée du nombre de coups enregistrés) due au phénomène de la radio-activité (Tableau 2). Cette

TABLEAU 2—REPRODUCTIBILITÉ DE LA MESURE RADIO-ACTIVE EFECTUÉE SUR UNE LARVE VIVANTE

Races	Poids des larves (mg)	Nombre total coups enregistrés			Moyenne	$\sqrt{\left(\frac{\Sigma\Delta^2}{n-1}\right)}$*	√(moyenne)†
		1ère mesure	2ème mesure	3ème mesure			
G	219,7	11 250	11 375	11 342	11 322	65	106
G	198,1	10 094	9 977	9863	9978	116	100
G	175,8	14 366	14 601	14 510	14 492	118	121
G	131,4	10 721	10 636	10 649	10 669	46	103
G	25,4	9100	9131	9234	9155	70	96
F	103,2	17 842	18 141	18062	18 015	155	134
F	93,7	15 336	15 616	15 832	15 595	249	125
F	60,9	9822	9797	9470	9696	196	98

* Erreur standard sur une mesure calculée d'après la variabilité des résultats obtenus pour une même larve.

† Erreur statistique sur une mesure due aux hasards des désintégrations nucléaires.

première mesure est appelée A et résulte de la moyenne de trois comptages successifs qui comprennent chacun environ 10 000 coups. Avant la détermination de cette première mesure A, les larves sortant du régime radioactif sont placées pendant 2 hr dans de la farine non radio-active.

Afin de vérifier si, en circulant dans la farine ordinaire, les larves se débarrassent complètement des particules radio-actives qui pourraient adhérer à leur tégument, nous avons fait sur dix insectes la vérification suivante: après avoir déterminé dans les conditions habituelles la radio-activité relative d'une larve qui a passé 2 hr dans de la farine non radio-active, nous immergeons cette larve dans de l'eau pendant quelques minutes, nous la retirons, la séchons à l'aide d'un papier filtre et un nouveau comptage est fait. Les résultats présentés dans le Tableau 3 permettent de conclure qu'il n'existe pas de différence significative entre les radio-activités mesurées avant et après le bain. A mesure donc exclusivement le ^{32}P contenu dans les tissus de la larve.

Après pesée et mesure de la radio-activité d'une larve vivante, cette dernière est placée immédiatement dans un creuset en porcelaine. La mesure du ^{32}P total est faite après incinération de l'insecte pendant environ 10 hr à une température

voisine de 500°C. Pour dissoudre le phosphate des cendres, nous employons 2 ml de HCl (0,05 N), agitons fortement avec une baguette de verre et ajoutons ensuite 1 ml d'eau. Nous prélevons 1,2 ml que nous plaçons sur trois disques métalliques de 24 mm de diamètre, ces disques reçoivent donc chacun 0,4 ml; l'évaporation se fait sur une plaque chauffante maintenue à basse température et

TABLEAU 3—VÉRIFICATION DE L'ABSENCE DE ^{32}P SUR LES TÉGUMENTS DES LARVES

Races	Poids des larves (mg)	Cpm d'une larve non lavée	Cpm d'une larve lavée
G	220,0	1688	1700
G	219,4	4044	4076
G	201,0	2822	2796
G	192,2	1969	1983
G	168,7	1954	1934
F	178,0	1944	1901
F	154,4	1759	1776
F	140,8	4032	4055
F	133,4	1057	1052
F	127,8	2317	2280

VOL. 4 1960

chacun des disques est ensuite placé sous le compteur qui a été utilisé pour mesurer la radio-activité de la larve vivante. La moyenne des valeurs obtenues pour les trois disques est la mesure *B*. La méthode a été vérifiée en ajoutant 1 ml de solution de ^{32}P à des larves non radio-actives placées dans des creusets en procelaine. Le Tableau 4 montre que la perte en radio-isotopes est d'environ 7 pour cent avec une

TABLEAU 4—RÉCUPÉRATION DU ^{32}P TOTAL AJOUTÉ A UNE LARVE NON MARQUÉE

Dénominations	Coups/min/ml				Récupération du ^{32}P		
	Disque no. 1	Disque no. 2	Disque no. 3	Moyenne	%	Moyenne %	$\sqrt{\left(\frac{\Sigma\Delta^2}{n-1}\right)}$
Contrôle	6900	6920	7038	6953			
Larve (161,0 mg)	6641	6416	6339	6465	93,0		
Larve (158,5 mg)	6542	6758	6369	6556	94,3		
Larve (155,5 mg)	6706	6246	6674	6542	94,1	93,2	2,2
Larve (135,0 mg)	6887	6527	6445	6620	95,2		(2,4%)
Larve (100,0 mg)	6442	6147	6072	6220	89,5		

erreur de 2,5 pour cent qui dépasse l'erreur statistique due au comptage; cette plus grande erreur est partiellement due à la préparation des disques pour les mesures de radio-activité.

Les 7 pour cent de ^{32}P non récupérés restent collés à la paroi du creuset. Cette adsorption est encore beaucoup plus importante quand le creuset n'est pas porté à 500°C. Nous plaçons 1 ml de solution radioactive dans deux creusets en porcelaine; le contenu du premier est évaporé sur une plaque chauffante à basse température, le second creuset est placé dans le four à 500°C. Dans les deux cas, le phosphate est repris avec HCl dilué, des disques préparés et un premier comptage effectué. Les creusets sont lavés à l'eau distillée puis placés tous deux dans le four. De nouveaux disques sont préparés de la même manière que précédemment

TABLEAU 5—ADHÉSION DU ^{32}P À LA PAROI DU CREUSET LORSQUE L'ÉVAPORATION DE 1 ml DE SOLUTION (25,778 c/m) SE FAIT A BASSE TEMPÉRATURE ET SA RÉCUPÉRATION APRÈS AVOIR PASSÉ CE CREUSET AU FOUR À 500°C

Creusets	Coups/min/ml		
	Premier comptage		Second comptage
	Evaporation a basse temperature	Traitement au four a 500°C	Creusets lavés rapidement et placés au four a 500°C
1	8981		11 403
2		24 675	942

et comptés. Les résultats obtenus (Tableau 5) indiquent très bien la forte adsorption du ^{32}P sur la paroi du creuset après une simple évaporation et la possibilité de sa récupération après un passage au four.

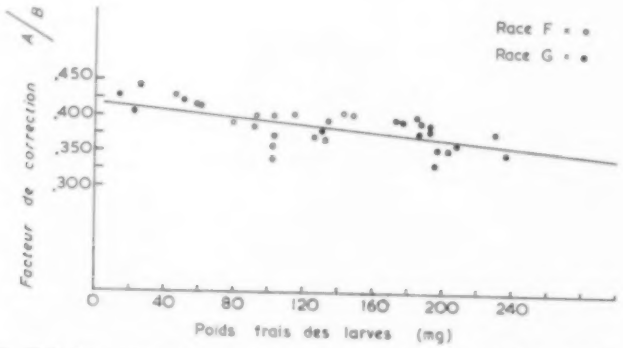


FIG. 2. Relation entre le facteur de correction A/B et le poids frais des larves. $Y = -.000258X + .42143$.

Le facteur de correction est A/B et il est entaché d'une erreur de l'ordre de 4 pour cent qui dépend principalement de B; nous ne devons pas ici tenir compte de la perte de 7 pour cent qui est une erreur systématique.

La courbe de la Fig. 2 montre la relation qui existe entre le facteur A/B et le poids frais des larves. L'analyse statistique a été effectuée à l'aides des formules de SNEDECOR (1946) et de FISHER (1949). Nous avons en premier lieu, fait le test de 'η' pour déterminer si Y est une fonction monotone de X. Le résultat étant positif, nous avons estimé le coefficient de corrélation $r = -0{,}61$. Ce coefficient est significatif par le test 't' de Student ($t = 5{,}5$). Le test de χ^2 ne décèle pas d'hétérogénéité significative entre les résultats expérimentaux et l'hypothèse linéaire ($\chi^2_{(4)} = 9{,}52$; $P < 0{,}05$). L'équation de la courbe de régression ($Y = mX + k$) a ensuite été estimée ainsi que le degré de précision de cette courbe par le calcul de 'Sy' (erreur standard de l'estimé). La valeur de Sy est de 5 pour cent et est du même ordre de grandeur que l'erreur sur le quotient A/B. Il semble donc raisonable d'assimiler à une droite la fonction $A/B = f$ (poids) dans les limites où elle est ici considérée.

DISCUSSION

On constate que le facteur A/B ne diminue que très peu quand le poids de la larve augmente.

Expérimentalement, on a vérifié que la rapport [($\sqrt[3]{}$poids)/longueur] reste constant pendant la croissance de la larve ce qui indique, puisque l'animal est sensiblement cylindrique, que ses proportions se maintiennent (Fig. 3). L'épaisseur

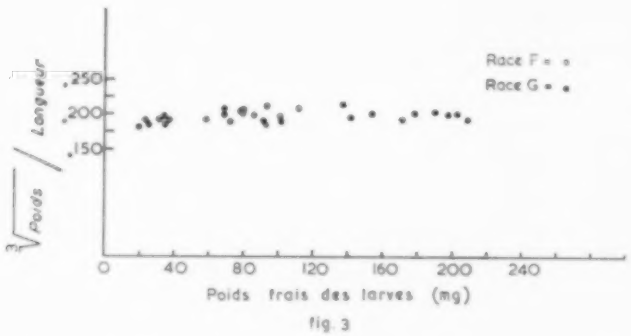


FIG. 3. Régularité du rapport [$\sqrt[3]{}$(poids)/longueur] pendant la croissance larvaire.

de la larve, paramètre dont dépend l'auto-absorption du rayonnement β et par conséquent le facteur de correction, varie donc comme la racine cubique du poids. On constate néanmoins qu'une augmentation d'épaisseur par un facteur de 1,35 (passage de 100 à 250 mg) ne s'accompagne que d'une variation de 10 pour cent du facteur de correction A/B.

Cette faible variation s'explique parce que nous travaillons dans la portion à faible pente de la courbe d'auto-absorption et que l'angle utile moyen pour le

comptage augmente avec le diamètre de la larve (Fig. 4) atténuant ainsi la diminution du rendement de la mesure due à une plus grande auto-absorption.

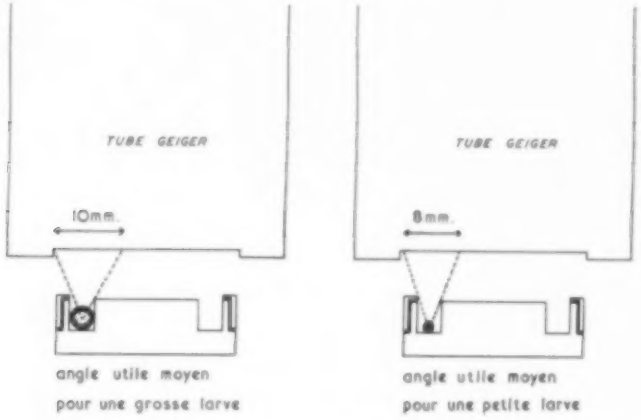


FIG. 4. L'angle utile moyen pour le comptage augmente avec le diamètre de la larve.

CONCLUSIONS

Nos résultats démontrent qu'il est possible de mesurer le ^{32}P total d'une larve vivante de *Tenebrio molitor* avec une erreur de ± 5 pour cent qui dépend essentiellement de l'erreur sur le facteur de correction appliqué. La très faible variation de ce facteur de correction en fonction du poids de la larve a été interprétée.

BIBLIOGRAPHIE

FISHER R. A. (1949) *Statistical Methods for Research Workers*. Oliver and Boyd, Edinburgh.

LECLERCQ J. (1950) Ecologie et physiologie des populations de *Tenebrio molitor* L. *Physiol. comp.* **2**, 161–180.

SNEDECOR G. W. (1946) *Statistical Methods Applied to Experiments in Agriculture and Biology*. Collegiate Press, Iowa.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 209 *to* 219. *Pergamon Press Ltd., London. Printed in Great Britain*

THE SALIVARY SECRETIONS OF A PLANT-SUCKING BUG, *ONCOPELTUS FASCIATUS* (DALL.) (HETEROPTERA: LYGAEIDAE)—II PHYSICAL AND CHEMICAL PROPERTIES

P. W. MILES*

Department of Zoology, University of Cambridge

(*Received* 15 *September* 1959)

Abstract—Histochemical tests indicate that the material which forms the stylet sheath contains a lipoprotein, is rich in tyrosine, and contains traces of tryptophan. Chitin and carbohydrate appear to be absent. There is no evidence that pectic substances can diffuse into the material from plant tissue. Sheath material is probably stabilized mainly by carbonyl–imino linkages, but there is evidence of additional aromatic bonding. The material shows similarities to the lipoprotein of the cockroach cuticle before the onset of final tanning and hardening, when it has been suggested that circular lipoprotein is 'self-tanned'. The watery saliva appears to contain mucoprotein.

VOL. 4 1960

INTRODUCTION

THE stylet track or sheath which remains when phytophagous Hemiptera feed on plant materials was at one time generally thought to be precipitated from plant cells by the action of the insects' saliva, and claims were made that stylet sheaths contained such substances as calcium pectate, callose, and tannin. SMITH (1933) reviewed the literature on the composition of sheath material and carried out numerous chemical tests from which, however, he concluded that the sheath produced by *Eutettix fabae* (Jassidae) and by *Stictocephala festina* (Membracidae) was wholly secreted by the insect and was composed of protein and chitin. He suggested that if any plant substances were also present, they diffused into the mass adventitiously before it hardened. FIFE (1932) and BENNETT (1934) meanwhile offered the final proof of the insect-origin of stylet sheaths when they reported that *Eutettix tenellus* secreted sheath material and even formed a stylet sheath when it fed on solutions through a paraffin membrane.

Evidence that the sheath material of *Oncopeltus* is a salivary secretion has already been presented (MILES, 1959b). The present paper describes an investigation into the chemical composition of this material, and reports the results of some additional tests on the separate watery saliva which this insect and various other Hemiptera are now known to secrete (DAY *et al.*, 1952; MILES, 1958, 1959a).

* Now at the Waite Agricultural Research Institute, University of Adelaide.

MATERIALS AND METHODS

The collection of the salivary secretions of *Oncopeltus* has been described previously (Miles, 1959b). Unless otherwise stated, tests were made on the fresh, unfixed sheath material. For tests on the watery saliva, the deposits left by the insect in sucrose–agar films were fixed by immersion for 24 hr in Lillie's neutral formol; the slides were then washed in running tap water for 2 hr to remove all traces of sucrose, and finally left in three ½-hr changes of distilled water.

Details of the histochemical tests used in this investigation can be found in Pearse (1953), Cole (1933), or Gatenby and Beams (1950) if no other source is given.

GENERAL PROPERTIES OF THE SHEATH MATERIAL

The mounds of sheath material secreted onto glass slides were colourless, hyaline, and moderately plastic. They could be pressed flat under a coverslip without fracturing, but a shearing pressure caused them to crumble. They usually showed birefringence around the central canal and along the surface in contact with the glass, and flattening caused general birefringence, but never of the intensity shown by silk.

The material was insoluble in water, organic solvents, and dilute acids and alkalis. In cold concentrated hydrochloric acid it swelled rapidly to about two to three times its original volume, but no further swelling or dissolution occurred within several days; it turned violet-pink after several hours, and the colour turned to yellow on subsequent exposure to ammonia. With concentrated sulphuric acid or nitric acid, it swelled in the same way but turned greenish yellow, and became orange when treated with ammonia. In concentrated alkalis it did not swell, but dissolved very slowly in the cold or rapidly at 160°C.

When immersed in sulphonephthalein indicators, it absorbed the dyes giving a colour indication with bromo-cresol purple and diethyl red of pH 5·8. The isoelectric point, estimated by the method of Yonge (1932), was pH 5·4 and was in good agreement with the methylene blue extinction value of about pH 5.

On heating, the material charred without melting and left no residue when ignited.

CHEMICAL COMPOSITION OF THE SHEATH MATERIAL

Protein tests

The material gave a strongly positive Millon reaction, Mörner's reaction, and tetrazonium reaction, the last also after treatment with performic acid to block tryptophan: these results indicate the presence of tyrosine. After the Hopkins–Cole–Adamkiewicz reaction, the material was green, but with a distinct purple tint in depth; it is therefore probable that tryptophan is present. The material dissolved if left for 12 hr in 10 per cent trypsin at pH 8, or for 24 hr in 10 per cent pepsin at pH 2, both at 37°C; specimens incubated with buffer alone did not. The material dissolved rapidly in cupri-ethylenediamine (see Coleman and Howitt, 1947); but when placed in 1 ml distilled water containing the tanning

agent benzoquinone, the masses rapidly turned reddish brown and became insoluble in proteolytic enzymes and in cupri-ethylenediamine. There seems little doubt that the material contains protein.

Carbohydrate tests

The material did not react positively with the Molisch test for carbohydrates. It stained with Schiff's reagent, even after treatment with bisulphite to block aldehydic groups (GLICK, 1949), but the colour faded within a week. On the other hand, after the PAS technique, a fast, permanent colour was produced. To discover whether this result indicated carbohydrate, the acetylation procedure was used. PEARSE discusses the acetylation of PAS-positive structures and decides that all become negative, but that carbohydrates alone become positive again after subsequent hydrolysis in alkali. With *Oncopeltus* sheath material, however, acetylation if anything enhanced staining by the PAS technique, and the colour was permanent; hence confirmation of carbohydrate was not obtained.

PEARSE lists only lipoproteins as being Millon-positive and PAS-positive but free of carbohydrate.

VOL. 4 1960

Lipid tests

The material used in these tests was produced by insects kept in a grease-free dish and was rinsed rapidly in chloroform as a further precaution against contamination with adventitious grease.

When treated with Sudan Black B by the method of PANTIN (1948), the material stained a pale blue-black. It also absorbed some colour from saturated solutions of Sudan III or Sudan IV, but unevenly and mainly on the exterior. This Sudan staining could be washed out in acetone and renewed several times.

The PFAS test for unsaturated lipids was negative.

When heated gently in concentrated nitric acid saturated with potassium chlorate (WIGGLESWORTH, 1933), the material dissolved with the evolution of gas and eventually some oily droplets appeared to be produced. When Sudan III was run onto the slide, some material at least stained strongly. However, the most convincing result with this test was obtained in the following manner. A large number of sheath masses were scraped into a glass thimble, one drop of the reagent was added, and the thimble heated gently until microscopic examination showed that the last traces of material were disappearing. Two drops of Sudan III were then added, and the surface of the liquid scanned. After a few minutes, unmistakable orange-red globules were found.

With the Liebermann–Burchardt test for sterols, the reaction was variable. In most cases, a violet-pink coloration was given at first, and in all cases, eventual shades from yellow through greenish-brown to bright green were obtained. Although the blue-green of cholesterol and its esters was not obtained, the results were similar to those described by BOSCOTT and MANDL (1949) with various other steroids.

After extraction with pyridine (Baker, 1946), the material was yellowish. No oily droplets were obtained on heating with chlorated nitric acid, and the Liebermann–Burchardt reaction was negative, i.e. no different from that obtained when the material was treated with sulphuric acid alone. On the other hand, the material appeared to stain as readily with Sudan stains as before extraction, and gave the same reactions with the various Schiff techniques.

It is evident that the staining of sheath material with the Sudan dyes is not significant and that the Schiff techniques give anomalous results with this material. However, the positive result with chlorated nitric acid before but not after extraction with pyridine indicates the presence of a lipid; and the fact that a Liebermann–Burchardt reaction, although not typical of cholesterol, was given only before extraction also suggests a lipid and may indicate that it is a steroid. These results are consistent with a lipid bound to the protein.

Significance of the Schiff reactions

Sheath material gave a strong Nadi reaction when tested within a few days of its secretion, indicating oxidizing properties, and this may explain the non-permanent colour given by Schiff's reagent after treatment to block aldehydes. However, the colour produced with the PAS technique was permanent and was evidently more than staining with re-oxidized fuchsin. Pearse states that, in biological materials, only polysaccharides, mucopolysaccharides, muco- and glyco-proteins, glyco- and phospho-lipids, and lipoproteins will be PAS-positive; yet with sheath material, the results of acetylation (to confirm carbohydrate) were anomalous, the Molisch test for carbohydrates and the PFAS test for unsaturated lipids were negative, and the PAS reaction was not affected by extraction with fat solvents. Further, experiments on the contents of the salivary glands of *Oncopeltus*, to be described in a later paper, showed the presence of some substances giving the typical reactions of carbohydrates with the PAS and acetylation techniques, and some giving the same reactions as sheath material; hence there was no reason to believe that faulty techniques had been used. Another possibility is that suggested by McManus (1948), who stated that some hydroxyaminoacids are oxidized by periodic acid and might therefore give the PAS reaction, but if this view is unjustifiable, as Pearse maintains, the PAS reaction of sheath material remains problematical.

Chitin test

Smith stated that the stylet sheaths which he examined in plant tissue did not dissolve in hot, concentrated potassium hydroxide and that the residue gave a positive chitosan test. However, *Oncopeltus* sheath material dissolved completely within 15 min in concentrated alkalis at 160°C, and it cannot therefore contain chitin.

Reaction with ruthenium red

Horsfall (1923) claimed that aphid tracks contained calcium pectate, since the material stained with ruthenium red after treatment with hydrochloric acid.

WITHYCOMBE (1926) obtained variable results using this test on Jassid tracks, whereas SMITH (1933) stated that ruthenium red stained the sheaths before acid treatment and that 'an acid-soluble substance, behaving like pectose, was indicated'. He believed, however, that if such a plant substance occurs in stylet tracks, 'it apparently dissolves and intermixes with the insect's secretion, which retains it on hardening'.

In view of these reports, the ruthenium red technique of JOHANSEN (1940) was used both on *Oncopeltus* sheath material secreted onto glass and on hand-cut sections of milkweed stem containing stylet tracks. The method depends on the conversion of pectic substances, by treatment with hydrochloric acid, to insoluble pectic acid, and its subsequent removal with dilute ammonium hydroxide. It is known that ruthenium red does not stain pectic substances alone (see GATENBY and BEAMS, 1950, § 1297 bis) and JOHANSEN directs that the same material be treated with the dye, and the stained areas compared, initially and after each treatment. *Oncopeltus* sheath material from both sources behaved similarly. It showed some affinity for the stain, although the affinity was destroyed

TABLE 1—PROPERTIES OF UNFIXED SHEATH MATERIAL

Reagent or Test	Reaction*		Inference
	Amino acid and protein tests		
Millon's	+		Tyrosine present
Mörner's	+		Tyrosine present
Tetrazonium after performic acid	+		Tyrosine present
Hopkins–Cole–Adamkiewicz	±		Tryptophan (trace) present
Proteinase	dissolves		Protein present
Benzoquinone	tans		Protein present
	Carbohydrate tests		
Molisch	0		Carbohydrate absent
PAS	+		Carbohydrate or lipid present
PAS after acetylation	+		Carbohydrate absent ?
	Lipid tests		
	BEFORE PYRIDINE EXTRACTION	AFTER	
Sudan stains	±	±	—
PFAS	0	0	—
Chlorated HNO_3	oily droplets	0	Lipid present
Liebermann–Burchardt	pink, turns green	0	Steroid ? present
	Other tests		
Chitosan	dissolves in KOH		Chitin absent
Ruthenium red (Johansen technique)	0		Pectic substances absent

* +, positive; ±, weak or variable; 0, negative.

by prior treatment with acid. When the acid treatment followed staining, however, further staining intensified the original colour, but it could not then be removed by subsequent treatment with alkali. It is evident, therefore, that the affinity of sheath material for ruthenium red is not an indication of the presence of pectic substances and cannot be considered as evidence that such substances diffuse into the stylet sheath secreted within plant tissues.

Probable composition

The tests described above and summarized in Table 1 indicate that the material which forms the stylet sheath is mainly protein, rich in tyrosine and with traces of tryptophan. There is some evidence of the presence of a lipid, possibly a steroid, linked to the protein. Tests for carbohydrate are either negative or anomalous, and chitin is absent. There is no evidence that pectic substances diffuse into tracks laid down in plant tissue.

STRUCTURAL STABILITY OF THE SHEATH MATERIAL

Reactions with swelling agents

Sheath material gels almost immediately it leaves the insect's stylets, even if discharged in aqueous media such as a 3 per cent agar gel containing 5 per cent sucrose; and drops of the material have been observed to set when bathed in the insect's watery saliva. The sheath material of leafhoppers will gel in dilute solutions of sucrose (Fife, 1932; Bennett, 1934). To investigate the type of linkage involved in the case of *Oncopeltus*, the procedure described by Brown (1950) was followed, with an additional treatment with cupri-ethylenediamine. In all cases, silk was also tested, and fragments of fingernail in the case of thioglycollate and sodium sulphide. The results are summarized in Table 2.

Table 2—Reactions of sheath material with swelling and dissolving agents

Reagent	Type of bond destroyed	Reaction
Boiling water	Van der Waals' forces	None in 2 hr
0·2 N HCl	Salt linkages	None in 24 hr
0·2 N NaOH	Salt linkages	None in 24 hr
6 M Urea	Salt linkages and H-bonds	None in 24 hr
Saturated LiI	Salt linkages and H-bonds	Turns brown, otherwise none in 24 hr
Cupri-ethylenediamine	Particularly CO–NH bonds	Dissolves rapidly
0·5 M Thioglycollate	Disulphide bonds	None in 24 hr
Saturated Na_2S	Disulphide bonds	None in 24 hr
10 N NaOCl	All, including aromatic covalent bonds	Dissolves

Silk thread swelled and dissolved in lithium iodide and in urea over 24 hr, and human keratin became distorted in thioglycollate and sodium sulphide, but in all these reagents, sheath material retained its dimensions. Both silk and sheath

material dissolved rapidly in sodium hypochlorite and almost instantaneously in cupri-ethylenediamine. The last forms a complex with NH groups, thereby splitting carbonyl–imino linkages, and it must be assumed that *Oncopeltus* sheath material owes its rigidity to these. According to COLEMAN and HOWITT, silk has only carbonyl–imino intermolecular bonds, and although it is stronger mechanically and shows stronger birefringence than *Oncopeltus* sheath material, silk is more readily attacked by swelling agents and by concentrated acids and alkalis. Thus some additional means of stabilizing sheath material may well exist.

Comparison with cuticular lipoprotein

There is no evidence of quinone tanning in sheath material, such as occurs in the protein of the insect cuticle (PRYOR, 1940a, b) and which appears to give rise to darkly coloured products (BRUNET and KENT, 1955); in fact, sheath material can be tanned with quinone *in vitro*, when it becomes dark red-brown and insoluble in cupri-ethylenediamine and proteinases. On the other hand, DENNELL and MALEK (1956) have suggested that the lipoprotein complex which forms in the developing cockroach cuticle achieves some degree of aromatic bonding by a process of 'self-tanning' (BLOWER, 1950) before the onset of final tanning and darkening; and *Oncopeltus* sheath material is similar in its resistance to chemical attack and its histochemical properties to this 'self-tanned' lipoprotein (Table 3).

VOL. 4 1960

TABLE 3—PROPERTIES OF SHEATH MATERIAL COMPARED WITH THOSE OF THE LIPOPROTEIN COMPLEX OF COCKROACH CUTICLE BEFORE TANNING AND DARKENING

Test or Reagent	'Cuticulin' layer of cockroach sclerites*	*Oncopeltus* sheath material
Mallory's triple stain	Red	Red-orange
Thionin blue	Blue	Pale blue
Iron haematoxylin	+	+
Sudan Black B	+	±
Liebermann–Burchardt	Blue green	Orange to green
Millon's	+	+
Mörner's	+	+
Argentaffin	+	+
Isoelectric point	pH 5·6	pH 5·4
HCl conc (hot)	Dissolves very slowly	Dissolves slowly
KOH saturated	Dissolves slowly	Dissolves slowly
Nadi	+	+

* From DENNELL and MALEK (1955a, b).

DENNELL and MALEK's hypothesis is based essentially on the resistance of the cuticular lipoprotein to solution in concentrated acids and alkalis, and on its argentaffin reaction, before final hardening. LISON (1953) states that, in biological materials, the argentaffin reaction is given by aminophenols, polyamines, dihydroxyphenols, and aldehydes; but the argentaffin reaction of sheath material

was not affected by incubation with bisulphite, which would block aldehydic groups, and Lison's confirmatory chromaffin test and other tests for diphenols (Vulpian, Gibb's, diazo) were negative. The lipoprotein complex of the developing cockroach cuticle also fails to give a positive test for diphenols; and although Lison specifically excludes tyrosine (when cold) from possible argentaffin substances, Dennell and Malek believe that oxidation of tyrosine hydroxyls in the cuticular protein may produce quinones *in situ* which condense with amino portions of other molecules (see Hackman, 1953) to give argentaffin substances. Such a process would be one of self-tanning. Positive reactions with Nadi reagent indicate that the oxidizing conditions required by this hypothesis obtain in both sheath material and the lipoprotein of the developing cockroach cuticle.

From the description given by Dennell and Malek, the lipoprotein of the cockroach cuticle before final hardening would seem to differ from *Oncopeltus* sheath material mainly in the status of the lipid moiety. The reactions of these materials suggest that there is less lipid and that it is more closely bound to the protein in sheath material; and since cuticular lipoprotein gives a blue-green colour in the Liebermann–Burchardt reaction (Dennell and Malek, 1955b), there may be a difference in the type of lipid involved. Nevertheless, the evidence on which Dennell and Malek base their 'self-tanning' hypothesis remains true of *Oncopeltus* sheath material, and since this material can easily be isolated in a pure state, it is possible that further examination of its properties may throw additional light on the chemistry of the changes which occur in the developing insect cuticle.

THE DUAL NATURE OF THE STYLET SHEATH

The mounds of sheath material secreted onto glass tend to react uniformly with stains and histochemical reagents; and in the foregoing account of its properties, the material has been considered as a homogeneous substance. However, when stylet sheaths laid down in plant tissue were treated with stain combinations, variations in staining reaction in different parts of the same track were observed.

There was no differentiation into an inner and outer zone as described by Zweigelt (1915), Davidson (1923), and Painter (1928). Painter figures a stylet sheath with a thin, uniform, tubular, inner zone, variably thickened on the exterior, and he states that these zones can be demonstrated with iron haematoxylin; but if *Oncopeltus* stylet sheaths in milkweed stem were cut sagittally, the sides of the hollow centre appeared as dark lines in some conditions of lighting, whatever the stain, and this was certainly a purely optical effect.

On the other hand, with safranin and light green or Flemming's triple stain, different parts of an otherwise apparently uniform sheath were clearly differentiated. There was no regularity about the differentiation, apart from a general tendency for the regions further away from the point of entry to stain with light green or orange G. In the absence of a counter stain, the whole material stained with safranin, and sections were therefore washed in alcohol and restained several

times to determine whether differentiation was due to true differences in the affinities of the regions or to variable washing out of safranin with counterstain. The differentiation was constant.

The significance of these observations is not clear. It is possible that the stains indicate the gradual changes which occur within the sheath after its secretion; e.g. with Nadi reagent, mounds of sheath material showed oxidizing properties immediately after secretion, which were gradually lost from the outer layers first. Also, in a later paper, evidence will be presented that the sheath is formed from the secretions of at least two separate lobes of the salivary glands, and that the contents of these lobes show different properties; hence it is possible that the proportions of these secretions vary in different parts of a stylet track, although such variation would tend to be masked in the compact masses of sheath material secreted onto glass.

THE WATERY SALIVA

The appearance of deposits of watery saliva when fixed in formol and stained with safranin and light green or with toluidine blue has already been described (MILES, 1959b). It is evident that at least two components are present, since with toluidine blue followed by dehydration in alcohol, the deposits appeared as dark-blue reticulations on a background of purple.

When slides were washed with water before formol-fixation, typical deposits could not be found, indicating that the secretion will disperse in water. On the other hand, careful examination sometimes revealed traces of the reticulations, but not of the non-reticulated material, suggesting that the two identifiable components may have different solubilities.

The salivary masses were not coloured by treatment with benzoquinone, but Millon's reagent stained them a faint pink. The faint Millon reaction suggests that the secretion is proteinaceous, and although there was no reaction with benzoquinone, tanning with this reagent is less likely after prolonged treatment with formol, which would block free amino groups and itself tan the protein.

The PAS procedure stained the secretion the normal positive magenta colour. Acetylation, although it did not destroy the reaction, resulted in a distinctly bluish PAS reaction, and alkaline hydrolysis after acetylation produced the same result. Although these reactions cannot be taken as definite evidence of the presence of carbohydrate, the purple staining with toluidine blue and the indication of the presence of protein suggest one of the mucoid substances. These are classified by PEARSE as hyaluronic and chondroitin sulphuric acids, acid and neutral mucopolysaccharides, and mucoproteins. The acid complexes may be distinguished by their γ-metachromasia (pink to red) with toluidine blue, their staining with alcian blue, and their ability to bind methylene blue below pH 4. When deposits of *Oncopeltus* watery saliva were treated with toluidine blue and air dried, they showed a variable reaction, from orthochromatic to γ-metachromatic, but the deposits did not stain with alcian blue nor, apparently, with methylene blue at any pH. Incubation of formol-fixed substrates with hyaluronidase did

VOL. 4 1960

not alter the properties of the secretion, confirming that hyaluronic and chondroitin sulphuric acids were absent. Finally, the solubility of the secretion in water, when considered in relation to the reactions described above, suggests that it contains mucoprotein.

Acknowledgements—This paper represents part of a thesis submitted for the degree of Ph.D. in the University of Cambridge; and my sincere thanks are due to my supervisor, Professor V. B. WIGGLESWORTH, and to Professor Sir JAMES GRAY for providing laboratory facilities. I am also grateful to Rhodes University, South Africa; to the trustees of the Southern Rhodesian State Lotteries; and to the trustees of the Charelick Salomon Scholarship Fund, Southern Rhodesia, for scholarships and grants which made the work possible.

REFERENCES

BAKER J. R. (1946) The histochemical recognition of lipine. *Quart. J. micr. Sci.* **87**, 441–465.

BENNETT C. W. (1934) Plant-tissue relations of the sugar-beet curly-top virus. *J. agric. Res.* **48**, 665–701.

BLOWER J. G. (1950) Aromatic tanning in the Myriapod cuticle. *Nature, Lond.* **165**, 569.

BOSCOTT R. J. and MANDL A. M. (1949) The histochemical demonstration of ketosteroids in adrenal tissue. *J. Endocrin.* **6**, 132–136.

BROWN C. H. (1950) A review of the methods available for the determination of the types of forces stabilizing structural proteins in animals. *Quart. J. micr. Sci.* **91**, 331–339.

BRUNET P. C. J. and KENT P. W. (1955) Observations of the mechanism of a tanning reaction in *Periplaneta* and *Blatta*. *Proc. roy. Soc.* (B) **144**, 259–274.

COLE S. W. (1933) *Practical Physiological Chemistry*. Heffer, Cambridge.

COLEMAN D. and HOWITT F. O. (1947) Studies on silk proteins—I. The properties and constitution of fibroin. The conversion of fibroin into a water-soluble form and its bearing on the phenomenon of denaturation. *Proc. roy. Soc.* (A) **190**, 145–169.

DAVIDSON J. (1923) Biological studies of *Aphis rumicis* Linn. The penetration of plant tissues and the source of the food supply of aphids. *Ann. appl. Biol.* **10**, 35–54.

DAY M. F., IRZYKIEWICZ H., and MCKINNON A. (1952) Observations on the feeding of the virus vector *Orosius argentatus* (Evans), and comparisons with certain other Jassids. *Aust. J. sci. Res.* (B) **5**, 128–142.

DENNELL R. and MALEK S. R. A. (1955a) The cuticle of the cockroach *Periplaneta americana* —II. The epicuticle. *Proc. roy. Soc.* (B) **143**, 239–257.

DENNELL R. and MALEK S. R. A. (1955b) The cuticle of the cockroach *Periplaneta americana* —III. The hardening of the cuticle: impregnation preparatory to phenolic tanning. *Proc. roy. Soc.* (B) **143**, 414–426.

DENNELL R. and MALEK S. R. A. (1956) The cuticle of the cockroach *Periplaneta americana* —V. The chemical resistance of the impregnating material of the cuticle, and the 'self-tanning' of its protein component. *Proc. roy. Soc.* (B) **144**, 545–556.

FIFE J. M. (1932) A method of artificially feeding the sugar-beet leafhopper. *Science* **75**, 465–466.

GATENBY J. B. and BEAMS H. W. (1950) *The Microtomist's Vade-Mecum*. Churchill, London.

GLICK D. (1949) Principles of enzymic histo- and cytochemistry. *Advanc. Enzymol.* **9**, 585–612.

HACKMAN R. H. (1953) Chemistry of insect cuticle—III. Hardening and darkening of the cuticle. *Biochem. J.* **54**, 371–377.

HORSFALL J. L. (1923) The effects of feeding punctures of Aphids on certain plant tissues. *Bull. Pa agric. Exp. Sta.* **182**.

JOHANSEN D. A. (1940) *Plant Microtechnique*. McGraw-Hill, New York.

LISON L. (1953) *Histochimie et Cytochimie animales, Principles et Méthodes*. Gauthier-Villars, Paris.

McMANUS J. F. A. (1948) The periodic acid routine applied to the kidney. *Amer. J. Path.* **24**, 643–648.

MILES P. W. (1958) Contact chemoreception in some Heteroptera, including chemoreception internal to the stylet food canal. *J. Ins. Physiol.* **2**, 338–347.

MILES P. W. (1959a) The secretion of two types of saliva by an aphid. *Nature, Lond.* **183**, 756.

MILES P. W. (1959b) The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera: Lygaeidae)—I. The types of secretion and their roles during feeding. *J. Ins. Physiol.* **3**, 243–255.

PAINTER R. H. (1928) Notes on the injury to plant cells by chinch bug feeding. *Ann. ent. Soc. Amer.* **21**, 232–242.

PANTIN C. F. A. (1948) *Microscopical Technique for Zoologists*. Cambridge University Press.

PEARSE A. G. E. (1953) *Histochemistry: Theoretical and Applied*. Churchill, London.

PRYOR M. G. M. (1940a) On the hardening of the ootheca of *Blatta orientalis*. *Proc. roy. Soc.* (B) **128**, 378–393.

PRYOR M. G. M. (1940b) On the hardening of the cuticle of insects. *Proc. roy. Soc.* (B) **128**, 394–407.

SMITH F. F. (1933) The nature of the sheath material in the feeding punctures produced by the potato leaf hopper and the three-cornered alfalfa hopper. *J. agric. Res.* **47**, 475–485.

WIGGLESWORTH V. B. (1933) The physiology of the cuticle and ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera); with special reference to the function of the oenocytes and of the dermal glands. *Quart. J. micr. Sci.* **76**, 269–318.

WITHYCOMBE C. L. (1926) Studies on the aetiology of sugar-cane froghopper blight in Trinidad—I. Introduction and general survey. *Ann. appl. Biol.* **13**, 64–108.

YONGE C. M. (1932) On the nature and permeability of chitin—I. The chitin lining the foregut of Decapod Crustacea and the function of the tegumental glands. *Proc. roy. Soc.* (B) **111**, 298–329.

ZWEIGELT F. (1915) Beiträge zur Kenntnis des Saugphänomens der Blattläuse und der Reaktionen der Pflanzenzellen. *Zbl. Bakt., Abt.* II **42**, 265–335.

VOL. 4 1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 220 *to* 228. *Pergamon Press Ltd., London. Printed in Great Britain*

THE RELATION OF OXYGEN CONSUMPTION TO AMBIENT OXYGEN CONCENTRATION DURING METAMORPHOSIS OF THE BLOWFLY, *PHORMIA REGINA*

HELEN D. PARK and JOHN BUCK

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda 14, Maryland

(*Received* 18 *September* 1959)

Abstract—At 1–5 days of pupal life in *Phormia regina* at 25°C, respiration becomes oxygen-limited at ambient oxygen concentrations between 15 and 10 per cent. In 1 per cent O_2 it is reduced to about one-fifth of the corresponding control levels. One and 5 day pupae tend to be somewhat more sensitive to hypoxia than other ages. Pupae exposed to pure nitrogen for 4 hr show a subsequent respiratory overshoot in air as compared with controls. Considering the overshoot as repayment of oxygen debt, the theoretical extent of repayment in 7 hr is 14 or 26+ per cent, depending on whether development is considered to stop or to continue during anoxia. The corresponding repayments in pure oxygen are 26 and 33+ per cent, suggesting that the rate of post-anoxic respiration in air is physically limited.

INTRODUCTION

THE relation between environmental oxygen concentration and rate of oxygen consumption is often a useful indication of an organism's ability to regulate its respiration, and of the relative importance of fermentative and aerobic processes in its overall metabolism. Although the relation has been investigated in a wide variety of invertebrates, there are very few studies on insects in which a full series of oxygen concentrations has been used, and only one, to our knowledge, dealing with metamorphosis. In that work, GAARDER (1918) concluded that during the period between 100 hr and 150 hr after pupation of the beetle, *Tenebrio molitor*, oxygen consumption is independent of oxygen concentration above 6 per cent. The fly *Phormia regina* was used in the present study because of its short pupal life and active respiration.

MATERIALS AND METHODS

In the present investigation, 'pupal life' was taken as beginning at the 'white pupa' stage, reached after the pre-pupa has ceased feeding, emptied its gut, and become immobile and contracted (about 6 days after egg laying at 25°C). It is taken as ending when the adult fly emerges from the puparium, which, at 25°C, occurs about 140 hr after white pupa formation. Actually, true pupal life (adult development) does not begin until the last larval moult, which in *Phormia*

takes place within the puparium about 20 hr after the formation of the white pupa (KEISTER, 1953). The time at which true pupal life ends (i.e. beginning of pharate adult development in the sense of HINTON, 1946) is unknown.

White pupae of 58 mg mean weight were collected at 8 a.m. from horse meat cultures maintained at approximately 25°C, and washed thoroughly. Except for the 'zero day' pupae, which were used immediately, they were then stored at 25°C. Oxygen consumption of 0-, 1-, 2-, 3-, 4-, and 5-day-old individual pupae was measured at 25°C by WARBURG's direct method using 15 ml flasks with 0·33 ml of 10 per cent KOH in the insets. During each experiment the oxygen consumption of the experimental pupae was measured in air from 9 a.m. to 11 a.m., then in a lower oxygen concentration from 11 a.m. to 1 p.m., and finally in air again from 1 p.m. to 3 p.m. Controls were on the same schedule except that only air flushes were used. With 1, 5, 10, and 15 per cent O_2, a 5-min flush was adequate, but in order to attain complete anoxia it was necessary to use specially purified commercial nitrogen and to prolong the flush to 10 min. At the end of the flush the venting cocks were closed while nitrogen still flowed through the manifold, and a smaller flow of nitrogen was then maintained until the manometers were set at the end of the 15-min equilibration period. Manometers were read at 15-min intervals during each 2-hr period and oxygen-uptake rates were calculated from the last six readings. To eliminate the possibility of effects carried over from prior exposures, a given pupa was used at only one age.

VOL. 4 1960

It was impracticable to make the necessary exposures to the six oxygen concentrations each day for six successive days, all on pupae from the same hatch of eggs. Therefore, three groups of at least five pupae each, all from the same hatch, were used each day, each group being exposed to a different oxygen concentration. The exposures were randomized so that all possible combinations of the six gas concentrations, taken three at a time, were used at least once with each pupal age. The total number of groups of pupae of each age exposed to each oxygen concentration varied from five to nine, i.e. there are never fewer than twenty-five pupae involved in any mean rate presented, and usually not fewer than thirty. Significance of intergroup differences in response was determined by Student's t test, the 0·05 level of probability being used. In all figures, the cap lines on the individual points represent standard errors.

RESULTS

Oxygen consumption in air of control pupae from zero through 5 days of age is shown in Fig. 1, the three points for each age representing the mean values for the three 2-hr periods of measurement. The curve has the characteristic U shape of insect respiration during metamorphosis (e.g. NEEDHAM, 1950; AGRELL, 1953; KEISTER, 1953) and shows that aerobic metabolism in the *Phormia* pupa passes, at about 2 days of age, through a minimum which is only one-third of its initial and terminal values. Except for somewhat lower zero and 5-day values, and slightly shorter duration, the curve agrees well with that which KEISTER (1953) obtained in the same species by following individuals throughout development.

In Fig. 2 can be seen the rates of oxygen uptake at each of the six pupal ages in the six concentrations of oxygen. Although at all ages the rate of uptake in 15 per cent O_2 tended to be lower than in the air controls, the reduction was statistically significant only in 1-day-old pupae. At 10 per cent and lower concentrations the depression of respiration was significant at all ages.

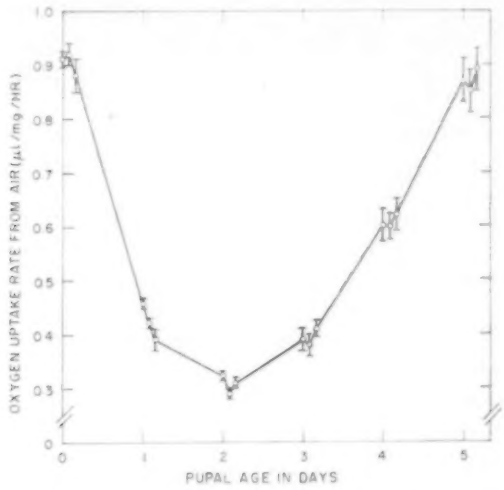


FIG. 1. Pupal oxygen-uptake rate in air at six ages.

If the respiratory rates of Fig. 2 are plotted as percentages of air rate against oxygen concentration there does not appear to be any striking difference in the respiratory embarrassment caused at the different ages. Thus the rate drop in 15 per cent O_2 ranges only from 1 to 7 per cent, in 10 per cent O_2 from 10 to 25 per cent, in 5 per cent O_2 from 28 to 46 per cent, and in 1 per cent O_2 from 71 to 86 per cent. Statistically significant differences do, however, exist, both between the sensitivities of various pupal ages to a given reduction in oxygen concentration and between the trends of respiratory depression in pupae of a given age in progressively lower ambient oxygen concentrations. For example (Fig. 2), the uptake rates of zero and 5-day-old pupae in air were almost equally high, yet the uptake of 5-day-old pupae was significantly lower than that of zero-day pupae in 1 per cent, 5 per cent, and 10 per cent O_2. Other differences are brought out when the data are plotted against pupal age (Fig. 3). For example, the oxygen-uptake rates of 2-day-old pupae in 1 per cent and 5 per cent O_2 are significantly less reduced than those of 1-, 4-, and 5-day-old pupae, and the rates of 3- and 4-day-old pupae in 10 per cent O_2 are less affected than those of 1- and 5-day pupae. In concentrations lower than 15 per cent there seem to be two periods of relative sensitivity to oxygen lack, one on day 1 and the other beginning on day 3 or 4 and reaching its maximum on day 5. Periods of relative

insensitivity occur at zero days and at 2–3 days, the latter period being close to the normal time of lowest uptake from air.

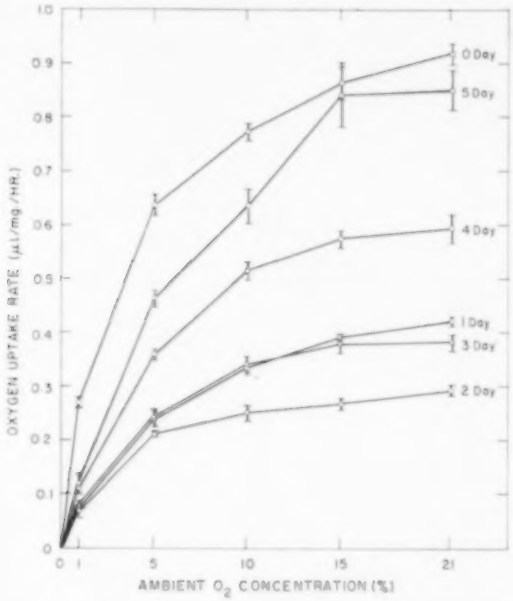


FIG. 2. Pupal oxygen-uptake rate in relation to age and ambient oxygen concentration.

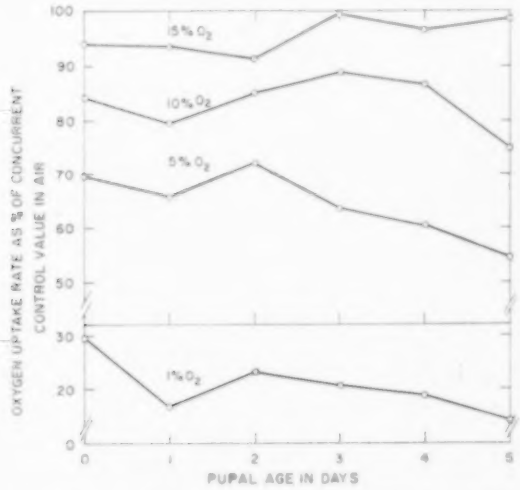


FIG. 3. Pupal oxygen uptake, as per cent of control rate, in relation to age and ambient oxygen concentration.

VOL. 4 1960

As shown in Fig. 4, a period in reduced oxygen may cause either a depression or enhancement of subsequent oxygen-uptake rate in air as compared with that of controls. The decreases are significant in 3-, 4-, and 5-day-old pupae after sojourn in 5 per cent O_2 and in 5-day-old pupae after 10 per cent O_2. None of the apparent increases in oxygen-uptake rate is significant. However, the several instances of slightly elevated rates after 2 hr exposure to nitrogen do suggest the possibility that some oxygen debt accrues during complete anoxia. In order to test this possibility further, experiments were carried out with longer exposure to nitrogen.

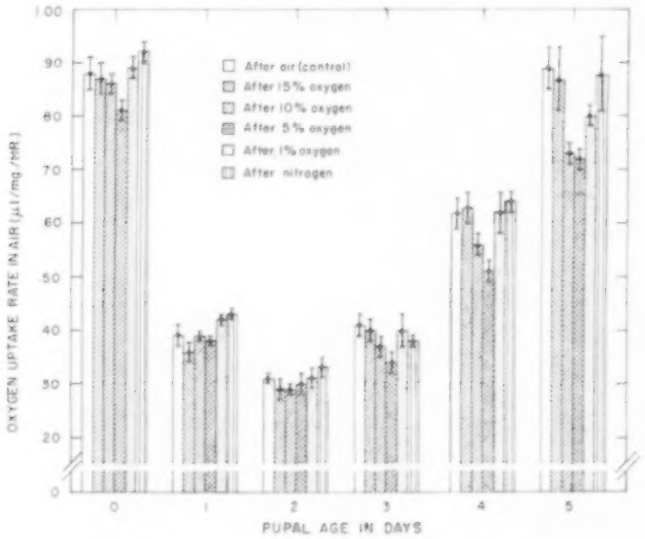


FIG. 4. Pupal oxygen-uptake rates in air following 2 hr exposure to a reduced oxygen concentration.

Seven experiments were done, each involving four groups of six to eight pupae each. After an hour's preliminary run in air the pupae were treated as follows: group 1—in air 4 hr, then in fresh air for an additional 7 hr; group 2—in air 4 hr, then in oxygen 7 hr; group 3—in nitrogen 4 hr, then in air 7 hr; group 4—in nitrogen 4 hr, then in oxygen 7 hr. One-day-old pupae were used because they have undergone the last larval moult and hence are operating on truly pupal metabolism, yet are respiring in the steepest part of the oxygen uptake curve, where their post-anoxic respiration should be, in the absence of debt repayment, significantly lower than their pre-anoxic respiration.

Respiration was found to be markedly elevated for some hours after removal of the pupae from nitrogen, but there is some question as to how to express this finding quantitatively. *A priori* it seems possible either that some pupal development proceeds during anoxia, or that progress halts. Some preliminary results on

Galleria (obtained by Dr. MARGARET KEISTER in our laboratory) suggest that anoxia interrupts development of the pupae and that upon return to air they simply prolong their pupal period by a corresponding amount. If the same situation holds in *Phormia*, post-anoxic oxygen uptake rate should be compared with that of controls that are 4 hr younger, rather than with that of controls of the same chronological age (but which are presumably developmentally older). The broken lines in Fig. 5 show the oxygen uptake rates of the experimental pupae on this basis. If, on the contrary, it be considered that development proceeds during anoxia and that post-anoxic respiration corresponds to that of contemporaneous controls rather than chronologically younger controls, the comparison would be between the control rates from hours 4–11 and corresponding experimental rates, as indicated by the solid lines in Fig. 5.

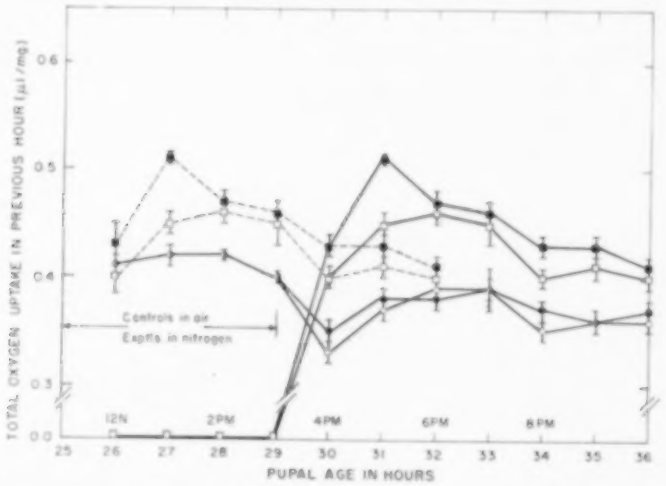


FIG. 5. Oxygen-uptake rates of 1-day-old pupae in air and oxygen following a 4-hr exposure to nitrogen. ■ Experimentals in oxygen; □ Experimentals in air; ● Controls in oxygen; ○ Controls in air.

Analysis of the data of Fig. 5 shows first, that the oxygen-uptake rates of the air and oxygen controls are not significantly different. Second, if the pupae are considered to have halted development during anoxia, the respiratory overshoot has disappeared by the end of the seventh hour after return to air. Third, if the pupae are considered to have developed contemporaneously with the controls, post-anoxic respiration is still significantly above the control level at the end of the seventh hour. Fourth, during the first 4 hr after removal from nitrogen the increase in oxygen-uptake rates over the control level is significantly higher in oxygen than in air.

The average debt contracted, taken as the mean cumulative oxygen uptake of the seven sets of controls over the 4 hr during which the experimentals were in nitrogen, was $1{\cdot}65 \pm 0{\cdot}02$ µl./mg. If the pupae exposed to nitrogen are considered

to have stopped development at the beginning of anoxia, the cumulative respiratory overshoot at the end of 7 hr, computed as the excess oxygen uptake in air after nitrogen over the oxygen uptake of 4-hr younger controls, was 14 ± 3 per cent in air, 22 ± 2 per cent in oxygen. These quantities are significantly different from both their control rates and from each other. If the experimental pupae are considered to have developed along with the controls the debt repayment at the end of 7 hr was 26 ± 2 per cent in air and 33 ± 2 per cent in oxygen, again significantly above the rates of contemporaneous controls and significantly different from each other. Furthermore, the trend of the curves indicates that some additional repayment would occur after the 7-hr period studied.

DISCUSSION

From the shapes of the curves showing oxygen-uptake rates with increasing concentrations of ambient oxygen (Fig. 2) we can conclude that during metamorphosis *Phormia regina* has little capacity for maintaining its respiratory rate in the face of lowered ambient oxygen concentration. One-day-old pupae show a significantly decreased oxygen-uptake rate in 15 per cent O_2, and all ages are limited by 10 per cent and lower oxygen concentrations. This dipterous pupa, therefore, stands in striking contrast to the larva of *Calliphora* (FRAENKEL and HERFORD, 1938) and to certain lepidopterous pupae in which oxygen uptake remains normal even in 1 per cent oxygen (BUCK and KEISTER, 1955).

That the differing sensitivities of the various pupal ages to oxygen deprivation reflect qualitative differences in underlying metabolic systems seems quite possible, particularly such a difference as that between the respiratory rates of zero day 'pupae' (essentially larvae) and 5-day pupae (essentially adults) in 1, 5, and 10 per cent O_2 (Fig. 2). However, in spite of, or perhaps because of, the availability of numerous chemical and enzymatic fluctuations during insect development (cf. HAUB and HITCHCOCK, 1941; AGRELL, 1953), it would be premature to attempt a specific association of any such changes with overall oxygen uptake.

One-day-old *Phormia* pupae join the small number of insects in which oxygen debt has been demonstrated directly, though the repayment is incomplete and spread out over a relatively long interval. Whether repayment would be greater with longer anoxia or at other ages, and the nature of the materials being oxidized during repayment, are questions of great interest, but, as emphasized by VON BRAND (1953), the theoretical degree of repayment is not likely to give any useful clue to biochemical events.

One possible explanation of the fact that post-anoxic respiration is greater in pure oxygen than in air is that in the 1-day-old pupa, at least, the oxygen concentration of air is insufficient to support all the potential respiratory metabolism. Inability to operate at optimum capacity can reasonably be attributed to the pupal spiracular apertures, which, on the one hand, are presumably set at an area which minimizes transpiratory water loss, and, on the other, lack any mechanical means of dilatation.

Our experiments give no clear bases for deciding whether development stops or is slowed during oxygen deprivation. The normal time of eclosion is sufficiently variable that the delay conceivably caused by the 4-hr exposure to nitrogen would be extremely difficult to detect. It is only possible to say that the apparent debt repayment is larger if the experimental and control pupae are compared on the basis of chronological age than on the assumption that development ceases during anoxia.

Two final mysteries of pupal respiration deserve mention even though no explanation is presently apparent. The first is the consistent dip in control respiration at the fifth hour after setup (age 30 hr in Fig. 5). Since the dip occurs in the first hour after the flush, the two events could conceivably be related. However, in a trial experiment using ten pupae, a decrease followed by a rise in respiration was also shown by 30-hr pupae in flasks which were not flushed. The consistent dip has no bearing on results and conclusions reported but does raise the possibility of some diurnal influence on respiratory rate with a minimum at 4 p.m. The second puzzle is the significant *depression* in oxygen-uptake rate in air following 2-hr exposure to 5 per cent O_2 at ages 3, 4, and 5 days (Fig. 4). In these pupae, in other words, release from a mild respiratory stress produces the opposite effect to what release from severe stress (2–4 hr in pure nitrogen) produces in pupa of 0, 1, and 2 days of age (Figs. 4, 5). This is particularly curious in that control respiration is rising steeply during the third to fifth pupal day (Fig. 1) so that the post-hypoxic rates in air might be expected to be, if anything, higher than before exposure to 5 per cent O_2.

VOL. 4 1960

SUMMARY

At 1–5 days of pupal life in *Phormia regina* at 25°C, respiration becomes oxygen-limited at ambient oxygen concentrations between 15 per cent and 10 per cent, and, in 1 per cent O_2, is reduced to about one-fifth of the corresponding control levels. One- and 5-day pupae tend to be somewhat more sensitive to hypoxia than other ages. Pupae exposed to pure nitrogen for 4 hr show a subsequent respiratory overshoot in air as compared with controls. Considering the overshoot as repayment of oxygen debt, the theoretical extent of repayment in 7 hr is 14 per cent or 26+ per cent, depending on whether development is considered to stop or to continue during anoxia. The corresponding repayments in pure oxygen are 26 per cent and 33+ per cent, suggesting that the rate of post-anoxic respiration in air is physically limited.

REFERENCES

AGRELL I. (1953) The aerobic and anaerobic utilization of metabolic energy during insect metamorphosis. *Acta physiol. scand.* **28**, 306–335.

VON BRAND T. and MEHLMAN B. (1953) Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some fresh water snails. *Biol. Bull., Woods Hole* **104**, 301–312.

BUCK J. and KEISTER M. (1955) Cyclic CO_2 release in diapausing *Agapema* pupae. *Biol. Bull., Woods Hole* **109**, 144–163.

FRAENKEL G. and HERFORD G. V. B. (1938) The respiration of insects through the skin. *J. exp. Biol.* **15**, 266–280.

GAARDER T. (1918) Über den Einfluss des Sauerstoffdruckes auf den Stoffwechsel—I. Nach Versuchen an Mehlwurmpuppen. *Biochem. Z.* **89**, 48–93.

HAUB J. G. and HITCHCOCK F. A. (1941) The interconversion of foodstuffs in the blowfly (*Phormia regina*) during metamorphosis—III. Chemical composition of larvae, pupae and adults. *Ann. ent. Soc. Amer.* **34**, 32–37.

HINTON H. E. (1946) Concealed phases in the metamorphosis of insects. *Nature, Lond.* **157**, 552–553.

HITCHCOCK F. A. and HAUB J. G. (1941) The interconversion of foodstuffs in the blowfly (*Phormia regina*) during metamorphosis—I. Respiratory metabolism and nitrogen excretion. *Ann. ent. Soc. Amer.* **34**, 17–25.

KEISTER M. L. (1953) Some observations on pupal respiration in *Phormia regina*. *J. Morph.* **93**, 573–587.

NEEDHAM J. (1950) The biochemistry of insect metamorphosis. *Biochemistry and Morphogenesis*, Part 2, Sect. 285. Cambridge University Press.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 229 *to* 234. *Pergamon Press Ltd., London. Printed in Great Britain*

CHLOROMYCETIN IN THE NUTRITION OF THE SILKWORM *BOMBYX MORI* L.—II. INFLUENCE ON DIGESTION AND UTILIZATION OF PROTEIN, FAT, AND MINERALS

M. B. SHYAMALA, K. SHARADA, MAYA G. BHAT, and J. V. BHAT

Fermentation Technology Laboratory, Indian Institute of Science, Bangalore 12

(*Received* 14 *October* 1959)

Abstract—The nature and extent of the influence of chloromycetin on larval digestion and utilization of the principal dietary constituents—the proteins, fats, and minerals—was studied. The antibiotic was shown to influence favourably the utilization of all the constituents studied. The results have been discussed in the light of these and other findings.

VOL.
4
1960

INTRODUCTION

DURING recent years a large volume of literature has accumulated on the subject of the growth-stimulating influence of small quantities of antibiotics in the food of farm and other animals. Various explanations of this have been offered: evidence has been adduced to show that antibiotics bring about beneficial changes in vitamin requirements and in the metabolism of protein, fat, and minerals, as well as in the intestinal microflora themselves. Detailed information on some aspects of the subject is available in several reviews (BRAUDE *et al.*, 1953; HEILMAN, 1953; STOKSTAD, 1954; NUTRITION REVIEWS, 1956).

Earlier work from this laboratory indicated that chloromycetin enhanced the growth and the fecundity of the silkworm (MURTHY *et al.*, 1954; SHARADA and BHAT, 1956) without significantly increasing the yield of silk though this consistently improved food utilization (SHYAMALA *et al.*, 1956). An examination of the intestines of silkworms to which had been administered chloromycetin, however, showed a more intense transaminase activity, though this could not fully explain the beneficial influence conferred by the antibiotic on the insect. Further experiments were therefore carried out with a view to studying the specific influence of this antibiotic on the metabolism of nitrogen and other constituents of a mulberry diet, such as crude fat and minerals.

MATERIALS AND METHODS

The experiments were conducted on fifth-instar silkworms, *Mysore* × *C. nichi* 1. Six lots of twenty worms each were assigned for each of the experimental groups, viz. (a) untreated, (b) acetone treated, and (c) treated with chloromycetin in acetone (200 μg/ml). The procedure for collecting samples of leaf and for collecting

excreta of the larvae was the same as that already described in an earlier paper (SHYAMALA *et al.*, 1956).

Collection of samples

(1) *Mulberry food.* Appropriate samples of leaf (both with and without chloromycetin) were collected from the food materials and their dry weights were recorded. Chloromycetin incorporation was effected by dipping the leaves in a solution of chloromycetin in acetone and allowing the acetone to evaporate in air. Various samples collected on different days were mixed in the proportion in which the leaves were supplied to the experimental worms, and their dry weights were also recorded.

(2) *Food left over and excreta.* These were collected once a day from each lot and finally pooled for the whole duration of the instar and weighed. The difference between the amount of leaf supplied and what was left over gave the data for leaf consumed; likewise, the difference between the quantities of consumed leaf and the amount of excreta gave data of leaf digested.

(3) *Larval samples.* The initial dry weights of the experimental larvae were computed by determining the dry weight of duplicate groups of twenty-five worms, each from the same population from which the experimental worms were taken. At the end of the fifth instar, larvae in three duplicate sets from each treatment were killed for the determination of dry weight and for other chemical analyses. The worms in the remaining three lots were allowed to spin cocoons. This silk was collected, dried, and weighed.

After the data had been collected for each of the three sets separately, the leaf and excreta samples from these were pooled, thus providing two samples of each of (1) mulberry food, (2) food left over, and (3) excreta for untreated, acetone-treated, and chloromycetin-treated groups. The pooled samples were powdered and preserved for further analysis.

The various samples collected were analysed for nitrogen, crude fat (ether extract), and ash. Carbohydrate determinations were not made, since all the samples were preserved after drying in the oven at 100–105°C. Nitrogen was estimated by the Kjeldahl method. Crude fat was determined by ether extraction in a soxhlet extractor for 16–20 hr. Ash was determined by the routine method (NICHOLLS, 1952).

When crude fat was estimated in leaf treated with acetone and acetone + chloromycetin, it was observed that there was a marked reduction in the crude fat content of the leaves left in the feeding trays. This resulted in recording very high values for fat utilization with acetone-treated groups, as compared to untreated controls. Hence, water was used as the solvent in a subsequent experiment. In this case, the fat content of supplied leaf was the same as in the two controls. Even though there was better growth and food utilization with water-treated groups, the disparity between the untreated and water-treated groups was not so great as to yield erroneous values.

RESULTS AND DISCUSSION

Tables 1–4 present the data for consumption, digestion, and the corresponding gain in the larvae for dry matter, nitrogen, ash, and crude fat respectively. The figures are the average of six values. Food utilization was calculated as the amount

TABLE 1—INFLUENCE OF CHLOROMYCETIN ON THE DIGESTION AND UTILIZATION OF DRY MATTER (IN g PER TWENTY WORMS)

	Untreated	Acetone treated	Chloromycetin treated	*P*
Consumed leaf	31·08	32·35	32·72	
Excreta	18·85	19·34	20·61	
Digested leaf	12·23	13·01	12·11	
Larval dry matter	5·857	6·412	7·087	
Percentage utilization	48·80 ± 0·42	49·62 ± 1·90	59·81 ± 1·64	0·01
Digestibility	39·29 ± 0·46	40·15 ± 0·82	37·03 ± 0·63	0·01

TABLE 2—INFLUENCE OF CHLOROMYCETIN ON THE DIGESTION AND UTILIZATION OF NITROGEN (IN g PER TWENTY WORMS)

	Untreated	Acetone treated	Chloromycetin treated	*P*
N in consumed leaf	1·458	1·529	1·531	
N in excreta	0·578	0·615	0·611	
N in digested leaf	0·880	0·914	0·920	
N in larvae	0·674	0·720	0·776	
Percentage utilization	76·83 ± 4·13	79·09 ± 2·16	85·01 ± 1·76	0·05
Digestibility	61·65 ± 1·49	59·65 ± 0·40	60·11 ± 0·55	not significant

TABLE 3—INFLUENCE OF CHLOROMYCETIN ON THE DIGESTION AND UTILIZATION OF MINERAL CONSTITUENTS (ASH IN g PER TWENTY WORMS)

	Untreated	Acetone treated	Chloromycetin treated	*P*
Ash in consumed leaf	3·979	3·847	4·144	
Ash in excreta	2·929	2·825	3·149	
Ash in digested leaf	1·050	1·022	0·995	
Ash in larvae	0·356	0·419	0·556	
Percentage utilization	36·4 ± 5·19	42·28 ± 3·37	56·44 ± 2·51	0·01
Digestibility	26·15 ± 2·07	26·51 ± 2·05	23·60 ± 1·12	not significant

of the constituents digested per 100 g of the mulberry consumed. The food utilization and digestibility data were analysed statistically and their standard errors calculated. The last columns in the tables give the level at which the results are significant, taking acetone as the control for chloromycetin treatment.

VOL. 4 1960

TABLE 4—INFLUENCE OF CHLOROMYCETIN ON THE DIGESTION AND UTILIZATION OF CRUDE FAT (IN g PER TWENTY WORMS)

	Untreated	Water treated	Chloromycetin treated	*P*
Fat in consumed leaf	1·030	0·950	0·942	
Fat in excreta	0·255	0·266	0·288	
Fat in digested leaf	0·775	0·684	0·654	
Fat in larvae	0·298	0·406	0·453	
Percentage utilization	38·33 ± 1·04	56·57 ± 4·196	67·49 ± 5·90	0·1
Digestibility	59·46 ± 3·75	50·33 ± 5·77	48·93 ± 9·25	

It may be seen from the tables that the digestibility of dry matter and fat was reduced in the chloromycetin-treated worms. However, the percentage utilization values show that the material utilized for building up larval tissue was significantly increased for all the constituents of mulberry leaf studied. Table 5 gives the percentage composition of the larvae.

TABLE 5—INFLUENCE OF CHLOROMYCETIN ON THE PERCENTAGE COMPOSITION OF LARVAE

	Untreated	Acetone treated	Chloromycetin treated
Dry matter	19·84	20·30	20·50
Nitrogen	11·94	11·53	11·44
Ash	6·74	7·03	8·27
Crude fat	21·60	24·44	23·34

Except for a slight increase in the ash content and the percentages of moisture, nitrogen and fat were not found to be affected by chloromycetin feeding.

TABLE 6—THE INFLUENCE OF CHLOROMYCETIN ON THE YIELD OF SILK

	Untreated	Acetone treated	Chloromycetin treated	*P*
Yield of silk (in g/20 worms)	2·503	2·604	2·702	
Silk produced for 100 g of larval dry wt.	36·0 ± 0·94	35·4 ± 0·47	33·5 ± 0·04	not significant
Silk produced for 100 g of larval nitrogen	55·56	57·82 ± 0·90	54·16 ± 0·09	0·01

In Table 6, the yield of silk and the percentage of larval nitrogen appearing as nitrogen in silk are given. It may be observed that whereas only 54·16 per cent of the larval nitrogen was utilized in the synthesis of silk in chloromycetin-fed larvae, 57·8 per cent conversion occurred in the acetone controls. Though the

difference is small, it is statistically significant. This finding is in support of the earlier claim made by MURTHY *et al.* (1953) that more preference is shown towards the building up of tissue material than towards increased formation of silk.

The results presented clearly show that under the influence of chloromycetin there was a more efficient utilization of the nitrogen (7 per cent), mineral constituents (28 per cent), and crude fat (20 per cent). The percentage composition of the larval tissues also shows that there was an increased deposition of minerals in the larvae treated with chloromycetin (Table 5).

The better utilization of the digested food was also found to be associated with an increased activity of the digestive enzyme proteinase of the gut (unpublished data) and by an increased concentration of vitamins in the larval tissue (SHYAMALA and BHAT, 1959).

Another aspect which was also brought to light during the course of investigations was that chloromycetin could be broken down by the silkworm intestines both *in vivo* and *in vitro*. *P*-amino benzoic acid and *p*-nitrobenzaldehyde could be detected in the degradation products. It was found that *p*-nitrobenzaldehyde, though it did not have any antibacterial action, could stimulate growth when given in small quantities (SHYAMALA and BHAT, 1959).

VOL. 4 1960

Further, chloromycetin administration was found to alter the intestinal microflora of the silkworm in that there was a general reduction in the bacterial population. Micrococci, streptococci, and flavobacteria were reduced in number with concomitant increases in the number of coliforms (unpublished data).

Studies of growth stimulation by chloromycetin indicate that the effect of its administration is reflected in several metabolic phases. But, whether the overall stimulation of metabolism is preceded by a primary influence of the antibiotic on a single metabolic step in the organism, or whether several processes in the system are simultaneously affected to give the observed cumulative effect, is still an open question. However, one point that has made itself fairly clear during these investigations is that chloromycetin does not manifest its favourable effect through the mediation of the intestinal microflora alone. It also gives rise to *p*-nitrobenzaldehyde which, though it is not antibacterial in nature, stimulates growth of the insect.

Acknowledgements—The authors wish to thank Miss S. SHARADAMMA for her assistance during the course of this investigation. Our thanks are due to the Central Silk Board, Government of India, for financial assistance and to the Director, Indian Institute of Science, for his keen interest.

REFERENCES

BRAUDE R., KON S. K., and PORTER J. W. G. (1953) Antibiotics in nutrition. *Nutr. Abstr. Rev.* **23**, 473–496.

HEILMAN F. R. (1953) Antibiotics. *Annu. Rev. Microbiol.* **7**, 219–244.

MURTHY M. R. V., SHANKARANARAYANA D., and SREENIVASAYA M. (1954) Role of chloromycetin in the nutrition of the silkworm *Bombyx mori* Linn. *J. sci. industr. Res.* **13** B, 331–335.

Nicholls J. R. (1952) *Aids to Analysis of Food and Drugs* (7th ed.). Baillière, Tindall and Cox, London.

Nutrition Reviews (1956) Effects of antibiotics in the ruminant. *Nutr. Rev.* **14**, 24.

Sharada K. and Bhat J. V. (1956) Effect of chloromycetin and glycine on the growth and production of silk by *Bombyx mori* L. *J. Indian Inst. Sci.* **38**, 136–147.

Shyamala M. B. and Bhat J. V. (1955) Effect of chloromycetin supplementation on transaminase activity of the silkworm *Bombyx mori* L. *J. sci. industr. Res.* **14** C, 97–99.

Shyamala M. B. and Bhat J. V. (1959) Chloromycetin in the nutrition of the silkworm *Bombyx mori* L.—I. Influence of the degradation products in nutrition. *Indian Inst. Sci. Golden Jubilee Res. Vol.*, pp. 222–230.

Shyamala M. B., Murthy M. R. V., and Bhat J. V. (1956) Effect of chloromycetin on food utilization by the silkworm *Bombyx mori* L. *J. Indian Inst. Sci.* **38**, 177–185.

Stokstad E. L. R. (1954) Antibiotics in animal nutrition. *Physiol. Rev.* **34**, 25–51.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 235 *to* 257. *Pergamon Press Ltd., London. Printed in Great Britain*

ON THE TISSUE ISOLATED IN SOME OF THE LARVAL APPENDAGES OF *SIALIS LUTARIA* L. AT THE LARVAL–PUPAL MOULT

B. J. SELMAN

Department of Zoology, University of Bristol

(*Received* 1 *November* 1959)

Abstract—Blood and epidermal cells are isolated in the old cuticle during the larval–pupal moult and are shed with that cuticle. The cells are left behind in the isolated fluid, which is a mixture of moulting fluid and haemolymph. No cells have been observed in the moulting fluid at any other moult. The number of cells cut off seems to be directly proportional to the size of the larva and hence to the size of the structure withdrawn. The blood cells in the isolated fluid are capable of clotting and forming tanned wound plugs at a time when the haemolymph of the pupa scarcely clots. The activity of the blood cells within the isolated fluid declines with time.

During the retraction of the epidermis in the gills, a new cuticle, or moulting membrane, is continuously secreted. Where this is exposed to activated moulting fluid it is dissolved immediately before the larval–pupal ecdysis except for the innermost layer, secreted immediately before the production of the new pupal cuticle. The latter layer envelops the whole body of the insect and seems to be homologous to the ecdysial membrane of other insects. The thick new cuticle within the gills does not appear to have any distinct functional significance for the pupa beyond protecting the retracting gill tip.

VOL. 4 1960

INTRODUCTION

IT has recently been found that in a few insects, blood and epidermis may be isolated in the old cuticle during moulting and shed with that cuticle. In the Tipulid genus *Lipsothrix*, HINTON (1955) found that at the pupal–adult moult about 1500 epidermal cells were isolated from the living insect in the lumen of each spiracular gill of the pupa. A cuticular membrane, called the basal occluding membrane, is secreted across the base of the gill. The tissue isolated in the spiracular gill does not degenerate until some time after the adult has shed the pupal cuticle. After the formation of the basal occluding membrane, the tissue in the gill is separated from the tissues in the body of the insect by two cuticles—the occluding membrane and the cuticle of the body wall of the adult—between which is the moulting fluid. In a later study (HINTON, 1957), it was shown that blood and epidermis are isolated in the spiracular gills of a large number of different Tipulids. The isolated tissue in *Lipsothrix*, *Taphrophila*, and some other genera is competent to repair wounds made in the spiracular gills throughout the pharate adult period and even for some days after the adult has shed the pupal cuticle. BURSELL (1958) has found that at the pupal–adult moult of the

tsetse fly epidermal cells remain attached to the first thoracic spiracle and thus become isolated in the moulting fluid.

It has not been previously noted that at the larval–pupal moult of *Sialis lutaria* L. blood and epidermis is isolated in the terminal filament, gills, and antennae of the cuticle that is to be shed. In this paper the moulting process is described together with the events that result in the isolation of tissue in the appendages. The possible functional significance of the isolated tissue is discussed.

MATERIALS AND METHODS

Collection and preparation of pharate pupae

Sialis lutaria larvae were collected at Abbots Pool near Bristol and from the Avon and Kennet canal between Limpley Stoke and Bathampton. Larvae in the early stages of pupation could be dug out of the banks in both of these localities in late March and early April. Final instar larvae collected from December to March would pupate in the laboratory at 19°C within 8–30 days. The later the larvae were collected in the field the sooner they pupated in the laboratory. Final instar larvae collected in the autumn were stored in a cold room at 10°C for 14–28 days. If the larvae were then warmed to 19°C they would begin to pupate in 5–8 days. In summer the ligaturing and chilling methods of GEIGY (1948), GEIGY and OCHSÉ (1940a, b), OCHSÉ (1944), GEIGY and RAHM (1951), and RAHM (1952) were used to obtain pupae. By ligaturing off the head of a larva, premature metamorphosis of the thorax and abdomen can be induced. Larvae of any instar can be used, but the percentage of successes increases with the age of the larva. After the larvae were ligatured for 5 days all moults were larval–pupal; before 5 days all moults of early instar larvae were larval–larval. The larval–pupal moult occurred within 35 days after ligaturing or not at all (RAHM, 1952). Ligaturing experiments with final instar larvae were found to be the most successful. A cold spell at 4°C reduced the time taken before moulting by ligatured final instar larvae. Thus, final instar larvae could be induced to pupate by these methods from August to March. Great losses of ligatured larvae were caused by fungi. The losses were completely eliminated by placing the newly ligatured larvae on filter papers moistened with 0·01 per cent $CuSO_4$ solution. Pupae obtained from ligatured early instar larvae tend to be incompletely developed, and they were not used. To eliminate possible sources of error, normal pharate pupae were used whenever possible. When pupae obtained from ligatured larvae were used, the results were always checked against those subsequently obtained from pharate pupae produced by unligatured larvae.

Examination of the tissue isolated in the appendages

The pharate pupae were first anaesthetized with carbon dioxide. The effect of carbon dioxide on the behaviour of the blood cells in the samples was checked against samples obtained from the same larva previously chilled on a block of ice. Since no difference was detected, carbon dioxide was used. To obtain the isolated

fluid, the tip was cut off well beyond the distal end of the retracting epidermis of the gill. The gill tip was transferred to a 0·8–1·0 mm thick glass slide which had been cleaned thoroughly to remove all traces of grease. Cleaning is most important as any trace of grease on a slide will greatly accelerate clotting of the blood cells. This may occur before the sample can be examined. The tip was then pressed with a needle to squeeze its contents onto the slide. The contents were quickly covered with pure liquid paraffin and observed using an oil immersion objective. No cover glass was used as blood does not clot at the paraffin–fluid interphase, but only at the glass–fluid interphase. This slows down the rate of clotting. Squeezing out the fluid before covering with liquid paraffin gives a thin flat film. If the liquid paraffin is added before the fluid is extruded from the gill, a spherical drop of fluid is obtained. This is useless for phase contrast observations. Only the cut-off tips of the gills or terminal filaments were used to exclude the possibility of the contamination of the fluid by haemolymph or blood cells from the body cavity of the pharate pupa. This can easily occur, for upon cutting off the gill tips, the haemolymph can burst open the tip of the retracting epidermal cells. This results in contamination of the remaining fluid with both blood cells and epidermal cells from within the body of the pupa. A further advantage of the method is that the gill tip can be transferred to the slide still attached to the cutting edge of the knife. This seals off the gill tip and prevents the loss of fluid and the exposure of the latter to the air until liquid paraffin can be added to cover the sample.

VOL. 4 1960

The fluid films were observed using a Cook, Troughton, and Simms phase contrast microscope with a sub-stage annulus. This was fitted with a dark green filter.

Histology

At each stage in the retraction of the epidermis the cut-off appendages and whole abdomens were fixed. These were either stained and mounted whole or embedded in paraffin wax and sectioned. Fixation of the gills proved to be most difficult. During the retraction of the epidermis the cuticle is highly resistant to the rapid penetration of fixatives. Only by heat fixation at 55°C was it possible to fix the cells within the fluid before their disintegration. Fixatives in low concentrations cause blood cells to clot. With hyaline haemocytes, clotting involves the discharge of cellular material into the surrounding medium. This process results in the total or partial disintegration of the cell. Only in very high concentrations can the most rapid fixatives, like methyl alcohol, fix the hyaline haemocytes before they clot. When the penetration rate of a fixative into a gill is slow, the concentration within the lumen builds up too slowly to prevent clotting. After heat fixation the abdomens were cut off and placed in Bouin's solution. Injection techniques proved valueless for fixing pharate pupae. Even when abdomens were cut open in fixatives, the tissues within the gills were not fixed quickly enough. This is because the gill tips are wholly or partially sealed off by thin cuticular lamellae secreted by the retracting epidermis.

Gills and abdomens to be sectioned were initially dehydrated in ethyl alcohol up to a concentration of 90 per cent and then finally dehydrated in absolute butyl alcohol. Methyl benzoate was used as the clearing agent. The last two reagents were used to reduce the degree of cuticular hardening, which results from the use of absolute alcohol and xylol. The specimens were then embedded in 56°C paraffin wax and cerosin at a reduced pressure of 6 cm Hg. The sections were stained with Heidenhain's, Ehrlich's, or Delafield's haematoxylin, counterstained with eosin, fast green, or orange C. The best results were obtained using the composite Mallory's triple connective tissue stain. The latter stain unfortunately fades with time. The fading was delayed by using Gurr's neutral mountant.

Vital staining

Larvae collected as they were leaving the water to pupate were injected with 0·1 cm^3 of a concentrated solution of either trypan blue or brilliant cresyl blue in 0·2 N saline approximately isotonic to the haemolymph. The variability in the osmotic pressure of the haemolymph is such that it is impossible to be completely accurate without doing a determination for each specimen. The injections were made at a time when there was no danger of damaging the retracting epidermis of the gills. These were made just behind the third thoracic tergite. After injection the larvae underwent a normal metamorphosis. The pharate pupae were then examined to determine whether or not dye particles were present in the isolated fluid. Trypan blue and brilliant blue were chosen for injection, as much of the dye remains as a fine suspension of particles and is easily observed within the living body. Dyes of other colours are not so readily seen.

OBSERVATIONS ON THE ISOLATED FLUID

When the fluid isolated in the gills and terminal filament at the larval–pupal moult is examined by phase contrast microscopy, large numbers of cells are seen. The cells were indistinguishable from the blood cells in the haemolymph of the final instar larva. Upon extrusion from the gill tip, the isolated fluid was clear and mobile. The cells were suspended individually in the fluid and showed no signs of agglutination. In normal insects, metamorphosis to an emerged pupa takes approximately 7 days at 10°C. The activity of the cells slowly diminishes but they are still able to form a clot on the sixth day.

The isolated fluid from the lateral gills

The cells are highly refractive immediately after the removal of the fluid from the gills (Fig. 1 a). The cells then begin to clot. Clotting is completed in approximately 20 min (Fig. 1 a–d). Fig. 1 shows four micrographs illustrating the course of the clotting of the cells. The majority of these cells are identical to the hyaline haemocytes of the final instar haemolymph. They clot by the production of hyaline veils, followed by the formation of vacuoles within the granular cytoplasm around the nucleus which first becomes clearly observable at this stage (Fig. 1 a–c). Immediately before the nucleus becomes clearly visible,

VOL.
4
1960

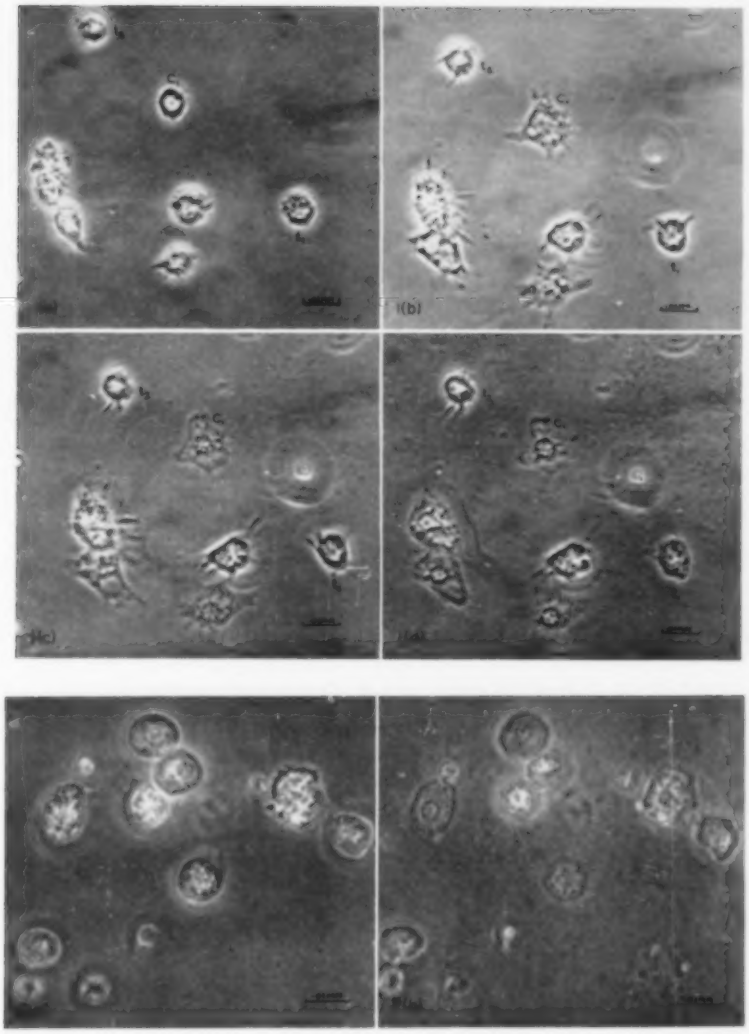
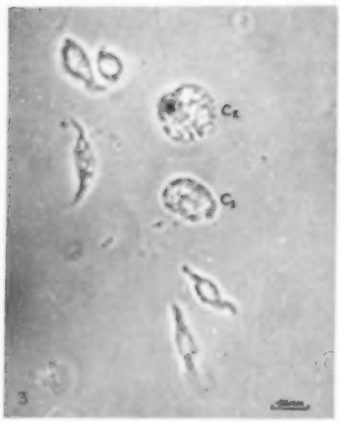
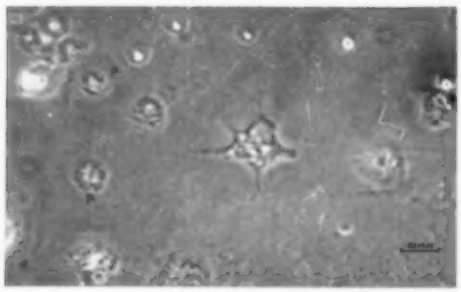
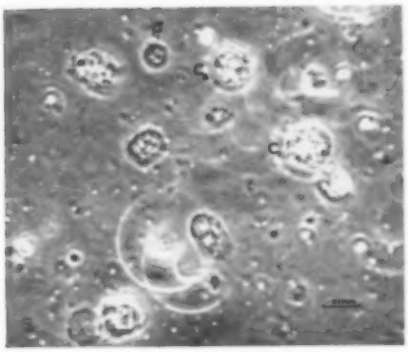


FIG. 1. A series of four micrographs taken over a period of 25 min, showing the clotting of blood cells within the moulting fluid of a pupa at the mid-pharate stage. The cells are all hyaline haemocytes.

FIG. 2. Two micrographs taken at an interval of 90 min, showing the clotting of the blood cells within the moulting fluid of a pharate pupa after the latter had been placed for 14 days in a cold room at 10°C.







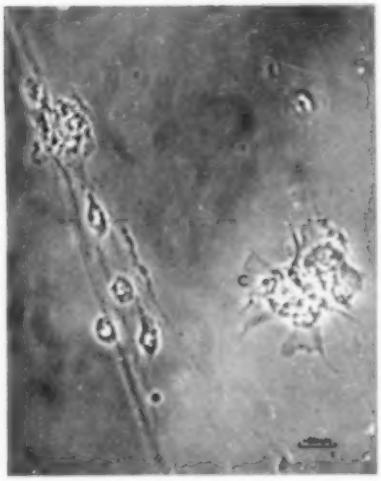


FIG. 3. The blood cells within the moulting fluid of the lateral gills of a late pharate pupa.

FIG. 4. The blood cells within the moulting fluid of the terminal filament of the same pharate pupa as in Fig. 3.

FIG. 5. Haemolymph removed from the labium of a late pharate pupa. The haemolymph is full of fat droplets and cells C_1 and C_2 seem to be rounded-off hyaline haemocytes heavily charged with lipoid material. The small cell P_1 is a proleucocyte. There is little or no clotting of the haemolymph.

FIG. 6. Blood cells within the moulting fluid of the terminal filament of a late pharate pupa. Note the cytoplasmic processes and the veils produced by the coagulocyte, hyaline haemocyte, C. The micrograph was taken before the discharge of cytoplasmic material into the moulting fluid.

there is a violent convulsion within the cytoplasm and cellular material is discharged into the isolated fluid. The latter begins to form a solid clot. The nucleus becomes easily visible and takes on the appearance of a fully clotted or fixed blood cell. The chromatin is arranged in granules, one or two at the centre, with a ring around the periphery of the nuclear membrane (Fig. 1 d). The result is the coagulation of the fluid (Fig. 1 d). Embedded in the coagulated fluid are two cells t_1 and t_2. These two cells have undergone little change during the process of clotting. In Fig. 1 (a) they would have been classified on account of their refractiveness and general similarity with the other cells in the photograph. Thus, it is difficult to distinguish c_1 in Fig. 1 (a) from t_1 in Fig. 1 (b). Cells t_1 and t_2 are much more refractive than fully developed granular haemocytes in blood samples from final instar larvae. Cells t_1 and t_2 seem to be intermediate between hyaline haemocytes and granular haemocytes.

In Fig. 2 (a)–(b) is shown the clotting of the isolated fluid in a specimen in which the retraction of the epidermis in the gills has been delayed after it had reached the half-way stage. This was achieved by placing the pharate pupa in a cold room at 10°C. At this temperature development is almost stopped. After 14 days the pharate pupa was removed from the cold room, and after 4 hr at 14°C an isolated fluid sample was taken from the gills. The cells were rounded and there was very little change in the shape of the cells during clotting. The degree of activity exhibited by the cells was observed to be much reduced. As a result of clotting, the cells lose much of their refractiveness (Fig. 2 b) and the nuclei assume the typical clotted or fixed appearance. Very little discharge of cytoplasmic inclusions was observed. However, the isolated fluid forms a highly granular clot (Fig. 2 b). The rate of clotting is much slowed down by the treatment, and takes up to 1 hr to arrive at a stage found after 20 min in a sample of fluid from an untreated pharate pupa at the same stage of metamorphosis. Examinations of a haemolymph sample from the leg of the same pharate pupa showed the blood cells to be normal in activity and appearance.

VOL. 4 1960

The isolated fluid of the terminal filament

After the larval–pupal moult, cells are found isolated within the terminal filament in even greater numbers than in the gills. However, differences are apparent. The epidermis of the terminal filament begins to retract 24–36 hr after the onset of retraction in the gills. The course followed by the epidermis in its retraction varied considerably from specimen to specimen, as described in the section on epidermal retraction. The cells isolated in the fluid were found to resemble the early pharate blood cells and not the blood cells of the final instar larva (Fig. 4). Many small proleucocytes and the early stages of hyaline haemocytes are present as are also cells which are already rounding off and becoming charged with lipoid material. This is in marked contrast to the sample of isolated fluid removed at the same time from a lateral gill of the same pharate pupa (Fig. 3). The cells in the isolated fluid from the gill were markedly less active than many of those in the terminal filament. Figure 6 shows more clearly the cells found

within the isolated fluid of the terminal filament. There is no difference between the cells of Fig. 6 and the blood cells found in haemolymph samples taken from pharate pupae with their gill epidermis two-thirds retracted. There are few fat droplets in the isolated fluid of the terminal filament. Small masses of degenerating tissue, frequently slightly-tanned, were found in many samples of isolated fluid from the terminal filament.

Haemolymph

Haemolymph removed from the labium of pharate pupae when the epidermis had fully retracted from the gills was full of highly refractive droplets (Fig. 5). These result from the breakdown of the larval abdominal fat during the last stages of gill retraction. After the breakdown of the larval fat body, the haemolymph is distinguishable from the isolated fluid. The spherical highly refractive cells (Fig. 5, c_1, c_2) are blood cells heavily charged with lipoid material. They readily stain black when Sudan Black is injected into the haemocoel 30 min before the removal of blood.

Within the blood of the pharate pupa a few granular haemocytes are to be found. These can be recognized by their often nearly triangular shape, dense granular cytoplasm, small nucleus unchanged during the clotting of the blood film, and marked lack of refractiveness. They are passive during clotting and become embedded in the clot. Granular haemocytes have never been found within the isolated fluid of the gills. By sectioning and taking blood samples during the development of the pharate pupa, it was found that the abdominal fat body begins to break down at the extreme posterior end when the gill epidermis is two-thirds retracted, i.e. approximately 2–3 days before the larval–pupal ecdysis. From the beginning of the time of the break-up of the fat body, highly refractive lipoid droplets are present within the haemolymph. During the period of the break-up of the fat body, the amoebocyte, hyaline blood cells become charged with lipoid material. They are clearly distinguishable from the cells isolated within the larval appendages before the break-up. At the end of the pharate pupal stage there are very few coagulocyte, hyaline haemocytes remaining in the blood. However, during the last stages of gill retraction, the proleucocytes begin to divide rapidly and increase in numbers. Few proleucocytes are found within the isolated fluid in the gills in contrast to the large numbers found within the body of the late pharate pupa.

Stained whole mounts of gills

Whole mounts of abdomens of middle and late pharate pupae proved to be most difficult to stain. The old larval cuticle stained with haematoxylin but the lumen of the gills, except at the extreme tips, remained unstained. This in marked contrast to final instar larvae in which the gills readily stain. Even when very little abdominal tissue was left it took up to 4 days for Delafield's haematoxylin to penetrate the gills. Similarly the differentiation and fixing of the stain was extremely slow and variable. Cells were found stained *in situ* in the isolated fluid within

the gill lumen. The fixed cells were of two types (Fig. 14, t, Co). Cells t_1 and t_2 are highly granular and have processes radiating out from the nucleus. They seem to correspond to the cells intermediate between the hyaline haemocytes and the granular haemocytes of the phase contrast micrographs (Fig. 1 a, t_1 and t_2). Cell i is intermediate between cells t_1 and t_2 and cells Co. Cell i has processes but no very dark granules in the cytoplasm. Cells of type Co have the appearance of heat-fixed hyaline haemocytes. The nuclei have most of the chromatin at the periphery, but the cytoplasm has been fixed before the typical coagulation patterns could form. In fixed gills and improperly heat-fixed gills, only a woolly mass remains in which scattered nuclei are embedded.

Vital staining

Four larvae which had just left the water were injected with trypan blue in saline isotonic to the haemolymph. At this time retraction of the gill epidermis had not begun. After the retraction of the gill epidermis, dye particles were clearly visible in the isolated fluid within the gills. The isolated fluid itself is stained pale blue. The dye particles could only be in the isolated fluid as a result of (a) epidermal cells left behind during retraction, or (b) isolated fluid at some time in continuity with the haemolymph.

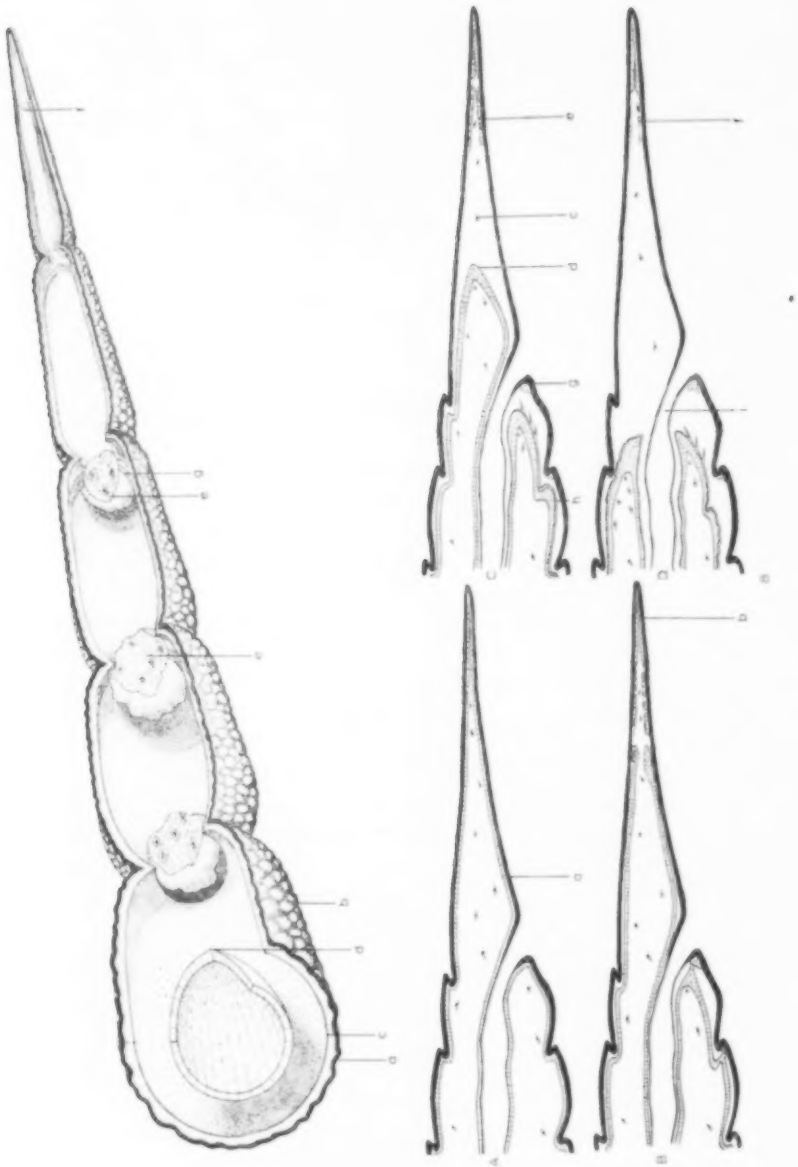
VOL. 4 1960

In the terminal filament, the isolated fluid is stained a pale blue and contains dye particles particularly near the distal tip of the filament. Injections of 0·05 cm^3 trypan blue and/or brilliant cresyl blue into pharate pupae with the terminal filament almost completely retracted resulted in dye appearing within the isolated fluid of the terminal filament but not of the gills. Injections of over 0·1 cm^3 were observed to burst open the retracting epidermis in the gills and also the terminal filament. No bursting open was observed with injections of 0·05 cm^3, the dye slowly diffusing into the terminal filament after approximately 20 min. This is after the heart has fully recovered from the effects of carbon dioxide used to anaesthetize the pharate pupae. Thus it seems unlikely that the small injections injure the pupal epidermis. At this stage of metamorphosis, it seems that in the terminal filament the isolated fluid is continuous with the haemolymph. The areas of continuity must be small. Pharate pupae burst open by large injections would still complete a normal metamorphosis.

The retraction of the epidermis within the abdominal filaments

Geigy and Ochsé (1940a, b) described the retraction of the tissue within the gills of *Sialis lutaria*. Their diagrams drawn from the unstained, living specimens show a continuous retraction of the tissue. No tissue was described as being left behind in the gills. Very similar diagrams were drawn by Ochsé (1944). Ochsé described in detail the retraction and reorganization of the gill stubs in the very late pharate pupal and free pupal stages. The early and middle stages of tissue contraction were hardly considered.

Observations on living specimens of large female pharate pupae show material left behind in the larval antennae, gills, and terminal filament. This material is



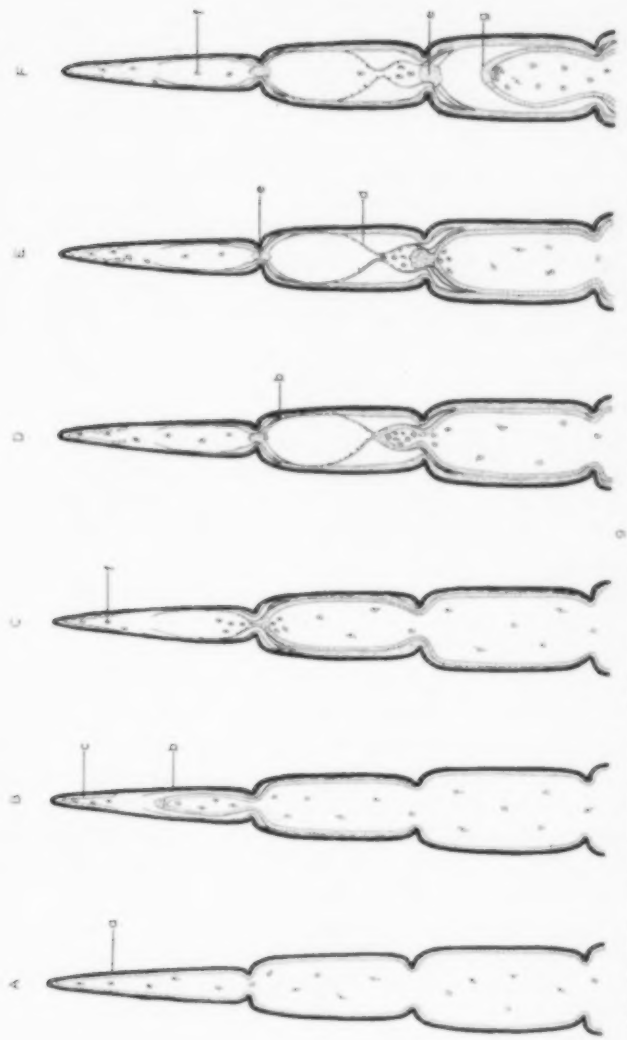


FIG. 7. Cut-away view of whole gill after tissue retraction but before activation of the moulting fluid. (a) Larval cuticle. (b) Wrinkled surface of larval cuticle. (c) Moulting membrane. (d) Tip of retracted epidermis. (e) Tissue plugs between segments. (f) Open distal end of moulting membrane. (g) Inner layers of moulting membrane.

FIG. 8. Diagram of stages of tissue retraction in the terminal filament, showing blood cells in the fluid. (a) Larval cuticle. (b) Epidermal cells isolated in the tip of the filament. (c) Blood cells isolated in the fluid. (d) Tip of retracting epidermis. (e) Basement membrane of isolated epidermal cells. (f) Cytolysing epidermal cells. (g) Cells isolated from the dorsal epidermis of the abdomen. (h) Pupal cuticle. (i) Rectum.

FIG. 9. Diagram of stages of tissue retraction in the gills, showing blood cells in the fluid. (a) Larval cuticle. (b) Moulting membrane. (c) Epidermal cells isolated in the gill tip. (d) Remains of cytolysed epidermal cells. (e) Tissue plugs between segments. (f) Blood cells isolated in the fluid. (g) Tip of epidermis retracting down third gill segment.

VOL. 4 1960

frequently darkly tanned. When the epidermis is half retracted in the gills, the retracting tip is pointed, not rounded as stated by GEIGY and OCHSÉ (1940). The tip was seen to fan out into the isolated fluid as a thin apparently structureless veil. At room temperature (19°C), the gill tissue of larvae brought into the laboratory immediately after constructing their pupal cells naturally in the field, is found to retract one gill segment per 24 hr. Retraction takes place in a series of five phases, with a pause between each.

The moulting membrane

One final instar larva collected in water in the field in late March was brought into the laboratory and placed in a water-filled vertical tube. The specimen died a week later midway through the pharate stage. The body was much distended and the larval cuticle could be clearly seen to be in two layers. The inner one was a complete layer except at the extremities of both the gills and the terminal filament. At the distal tips the inner layer was open (Figs. 7, 9). In final instar exuviae the second or inner layer can be separated from the outer. In sections of the abdomen of pharate pupae, the inner layer stains similarly to the endocuticle of the outer layer: a deep blue with Mallory's connective tissue stain or black with Heidenhain's haematoxylin. The endocuticle stains lightly. In sections of the gills the inner layer stains only a little darker than the endocuticle of the outer layer. Exceptions to this are the innermost layer in gill segment 1 and to a lesser extent the very innermost lamellae of the inner layer of gill segments 2 and 3, which stain darkly.

The second inner layer is not delaminated from the larval cuticle. It is not secreted until retraction of the epidermis from the old final instar larval cuticle begins. It is a new secretion at the time of moulting and the term moulting membrane (HINTON, 1958) will be used to describe it. This is synonymous to the term ecdysial membrane which has been used to describe much thinner but apparently homologous structures in the Cecropia silkworm (PASSONNEAU and WILLIAMS, 1953; RICHARDS, 1955), in *Ephestia* (KÜHN and PIEPHO, 1938), in bees (RICHARDS, 1952), and in the locust, *Schistocerca gregaria* (MALEK, 1958).

The epidermis of the pharate pupa continuously secretes cuticular material as it retracts from the gill cuticle. Thickening of the epidermis takes place before detachment, and the cuticle is pulled inward in places (Fig. 7). The moulting membrane is laid down in thin lamellae on the inner surface of the larval cuticle. The former fills in the concavities of the larval cuticle (Figs. 7, 15). Therefore the thickness of the moulting membrane in the gills is highly variable. As the epidermis retracts down the gill, further layers are added to the moulting membrane. When the gill epidermis was fully retracted, the thickness of the moulting membrane of twelve sectioned gills was measured (Table 1).

Measurements of the total thickness of the larval cuticle, plus the moulting membrane, showed a steady increase until the sixth day of the pharate pupal stage (Table 2). In Table 2 A, the slight reduction in thickness in the retracting gill of the late pharate pupa, is probably because of the onset of the activation of the

moulting fluid. The exuvium was from a specimen in which the second gill segment was walled off from the abdomen, and the isolated fluid was therefore not activated. As mentioned later, the isolated fluid is a mixture of moulting fluid and haemolymph and it can be activated as normal moulting fluid.

TABLE 1—MEASUREMENT OF THE MAXIMUM AND MINIMUM THICKNESSES OF THE MOULTING MEMBRANE IN TWELVE GILLS, 2 DAYS BEFORE THE LARVAL–PUPAL ECDYSIS

Segment of gill	Thickness of moulting membrane	
	minimum (average)	maximum (average)
Fifth	3 μ	4 μ
Fourth	5 μ	9 μ
Third	5 μ	12 μ
Second	7 μ	20 μ

VOL. 4 1960

In the abdomen the moulting membrane is most developed in the angle between the posterior ends of the abdominal terga and sterna, and the intersegmental membrane. The angles are formed by the shortening of the abdomen. They are filled with a large number of cuticular lamellae. All measurements of the moulting membrane were made along the lateral surface of the seventh abdominal segment just behind the base of the gill.

TABLE 2—THE THICKNESS OF THE CUTICLE AND THE MOULTING MEMBRANE OF (A) THE SECOND GILL SEGMENT AT INTERVALS DURING PUPATION, AND (B) THE SEVENTH ABDOMINAL SEGMENT AT INTERVALS DURING PUPATION

Time of measurement	A. Thickness of cuticle + moulting membrane	B. Thickness of cuticle + moulting membrane
Final instar larva	4–5 μ	16–18 μ
Early retracting gill pharate pupa	9–14 μ	13–23 μ
Mid retracting gill pharate pupa	13–15 μ	20–26 μ
Late retracting gill pharate pupa	13–14 μ	18–22 μ
Exuvia	13–15 μ*	5–8 μ

* A specimen with a walled-off second segment.

In Table 2, only the minimum thickness was recorded. The maximum thickness is impossible to estimate, as the lamellae of the moulting membrane are separated from one another, especially in the angles between the terga and the membrane between the terga.

In the abdomen measurements on sectioned exuvia (Table 3) showed that the thickness of both the moulting membrane and the cuticle of the final instar larva was reduced immediately before the larval–pupal ecdysis. This also occurs in some second gill segments (Table 4) but not in the third gill segment.

TABLE 3—THE THICKNESS OF THE LARVAL CUTICLE AND THE MOULTING MEMBRANE OF THE EXUVIA AFTER THE LARVAL–PUPAL ECDYSIS

Site of measurement	Larval cuticle		Moulting membrane	
	min	max	min	max
Abdomen	4 μ	5 μ	1 μ	2 μ
Second gill segment	3 μ	4 μ	3 μ	10 μ
Third gill segment	5 μ	18 μ	5 μ	15 μ
Fourth gill segment	4 μ	6 μ	4 μ	9 μ
Fifth gill segment	3 μ	3 μ	4 μ	4 μ

TABLE 4—THICKNESS OF CUTICLE AND MOULTING MEMBRANE BEFORE AND AFTER ECDYSIS IN (A) THE SECOND GILL SEGMENT, AND (B) THE THIRD GILL SEGMENT

Cuticular structure	Before activation of moulting fluid		After ecdysis	
	A	B	A	B
Moulting membrane	7 μ	5 μ	3 μ	5 μ
Final instar larval cuticle	4 μ	4 μ	3 μ	5 μ

During its retraction, therefore, the epidermis continuously secretes new cuticular material over the whole of the body except at the extreme tips of the filaments where tissue is left behind. The secretion of the moulting membrane is followed by secretion of the pupal cuticle. In the abdomen the pupal cuticle begins to be secreted on the third day of the pharate pupal stage, whereas the pupal cuticle is not secreted by the retracted lateral gill stubs until the sixth day, i.e. just over 24 hr before the larval–pupal ecdysis. During the last 24 hr before ecdysis, the thickness of both the final instar cuticle and the moulting membrane is greatly reduced (Table 3). The greatest increase in the thickness of the cuticle occurs in the second gill segment. In this segment the moulting membrane is secreted for a longer period than in any other part of the insect. The thickness of the cuticle in this segment is reduced immediately before the larval–pupal ecdysis in some specimens but not in others. The determining factor is whether or not the base of the second gill segment is walled off by degenerating epidermal cells and inner

layers of the moulting membrane. Where the plug is present there is no reduction in the thickness of the cuticle immediately before ecdysis. Where the plug is not present the thickness of the old larval cuticle, plus the moulting membrane, is reduced from 13 μ to 7·5 μ.

At the base of the third gill segment a plug is always present and reduction in the thickness of the cuticular layers does not occur. The thickness of the old larval cuticle and the moulting membrane of the third gill segment increases from 4–5 μ to 10–17 μ during the early part of the pharate pupal phase.

Wherever it is formed, the inner layers of the moulting membrane are clearly distinguishable from the endocuticle of the final instar larva. Immediately before ecdysis, that is during the last 24 hr of the pharate pupa, the endocuticle of the old larval cuticle and the outer layers of the moulting membrane are dissolved, leaving the apparently hardened inner layer of the moulting membrane. The moulting membrane is reduced to 0·1–2 μ in the abdomen and the first gill segment, and to 3 μ in the second gill segment when this is not separated from the abdominal isolated fluid.

If removed from water the final instar larva will very quickly lose moisture and die. It is possible that the moulting membrane may function to reduce water loss, and gills without a moulting membrane may dry up faster than gills with a moulting membrane.

VOL. 4 1960

Gills were ligatured at the base and cut off a final instar larva. Gills that had just completed retraction were also ligatured at the base and cut off a late pharate pupa. After drying the surface moisture on a filter paper, the gills were placed in a dehydration apparatus. This consisted of a small chamber connected to a phosphorous pentoxide water trap, the whole system being evacuated by a two-stage Edwards 'High Vac' oil pump to a pressure of 6 cm Hg. After 2 min the final instar gills flattened and dried. The gills of the late pharate pupa took 3 min to flatten and dry. Therefore it appears that the rate of dehydration of the gills is slightly reduced during pupation.

Retraction of the epidermis in the gills

Observations *in vivo* of the retracting gills show that the gill tissue retracts one gill segment at a time after which there is a pause. The cuticle of the gill ceases to be smooth and transparent at the beginning of gill retraction. The cuticle becomes crinkled except at the extreme tip, numerous indentations giving it a reticulate appearance. The gill is no longer completely transparent but slightly opaque. The epidermis retracts from the cuticle in the three distal segments of each gill. In the fifth and most distal segment the tissue retracts, leaving behind the epidermal cells at the extreme tip (Fig. 9, B). The break between the retracting epidermal cells and those left behind occurs approximately one-third of the distance down the fifth segment. The retracting tissue rounds off and eventually forms a tight-packed group of cells just distal to the constriction between the fourth and fifth gill segments. This group of cells becomes constricted at the base and appears to break down to a woolly mass. In whole mounts stained with

Delafield's haematoxylin, the woolly mass is seen to consist of degenerating cells fanning out into the moulting fluid from the retracting tip of the epidermal cells (Fig. 9, C). Sections show that before retraction the portion of the epidermis of the fifth gill segment that retracts, secretes a new cuticular layer or moulting membrane (Fig. 9, B) 3–4 μ thick. As the tissue retracts down the fourth segment, further layers of moulting membrane are secreted (Fig. 9, C–F). The inner layers of moulting membrane secreted during the retraction of the epidermis down the fourth segment are nipped in at the junction of the fourth and fifth segments (Fig. 9, C–D). They are secreted during the pause between the epidermal retraction in the fifth segment and the epidermal retraction in the fourth segment. After the retraction of the epidermis from the fourth gill segment, the opening of the lumen of the fourth into the fifth segment is seen to be blocked by a slightly opaque, woolly plug (Figs. 7, 9). The retraction of the epidermis from segments 2 and 3 is similar to that of segment 4. Plugs are seldom formed between the first and second gill segments. Plugs formed between the latter two segments are most common in the smaller male pharate pupae. After retraction is completed, examination of gills show nerves up to 75 μ long still attached to the base of the two large terminal sensilla.

The tips of the retracting epidermis in gill segments 3 and 4 were sectioned. At the tip, the epidermal cells round off and elongate inwards towards the centre of the gill lumen (Fig. 15). The epidermis of the gills of the pharate pupa appears to lack a basement membrane. The elongated cells normally cytolyse whilst still attached to the innermost or most recently secreted layer of the moulting membrane. The epidermal cells are occasionally detached and are free in the haemolymph before cytolysis begins. There is no evidence that the blood cells are responsible for the destruction of the epidermal cells. However, there is no doubt that the blood cells absorb the cellular débris set free in the haemolymph. Immediately distal to the retracting epidermal tip many cellular remnants are found (Fig. 15). These are both nuclear and cytoplasmic in origin. Thus the majority of the epidermal cells do not retract but are cytolysed. This gives a superficial appearance of retraction. After the epidermis has retracted from the fifth segment, the tip is closed off and the isolated fluid is never again continuous with the haemolymph in the body of the pharate pupa.

As already stated, the epidermis of the gill retracts one segment at a time with a pause between each phase of retraction. During each period of retraction the epidermal cells round off faster than they cytolyse. This results in the plug of tissue which is left behind at the junction between each gill segment (Fig. 9). The plug is the base of a pocket of epidermal cells nipped off just distal to the junctions of the gill segments. Each pocket encloses blood cells. When the epidermal cells cytolyse it appears that the blood cells together with a little haemolymph enter the isolated fluid.

Stained sections of gills show that all epidermal cells left behind by the retracting gill tissue do not cytolyse. This is particularly true of those that are immediately distal to the junction between each gill segment. Groups of epidermal cells are

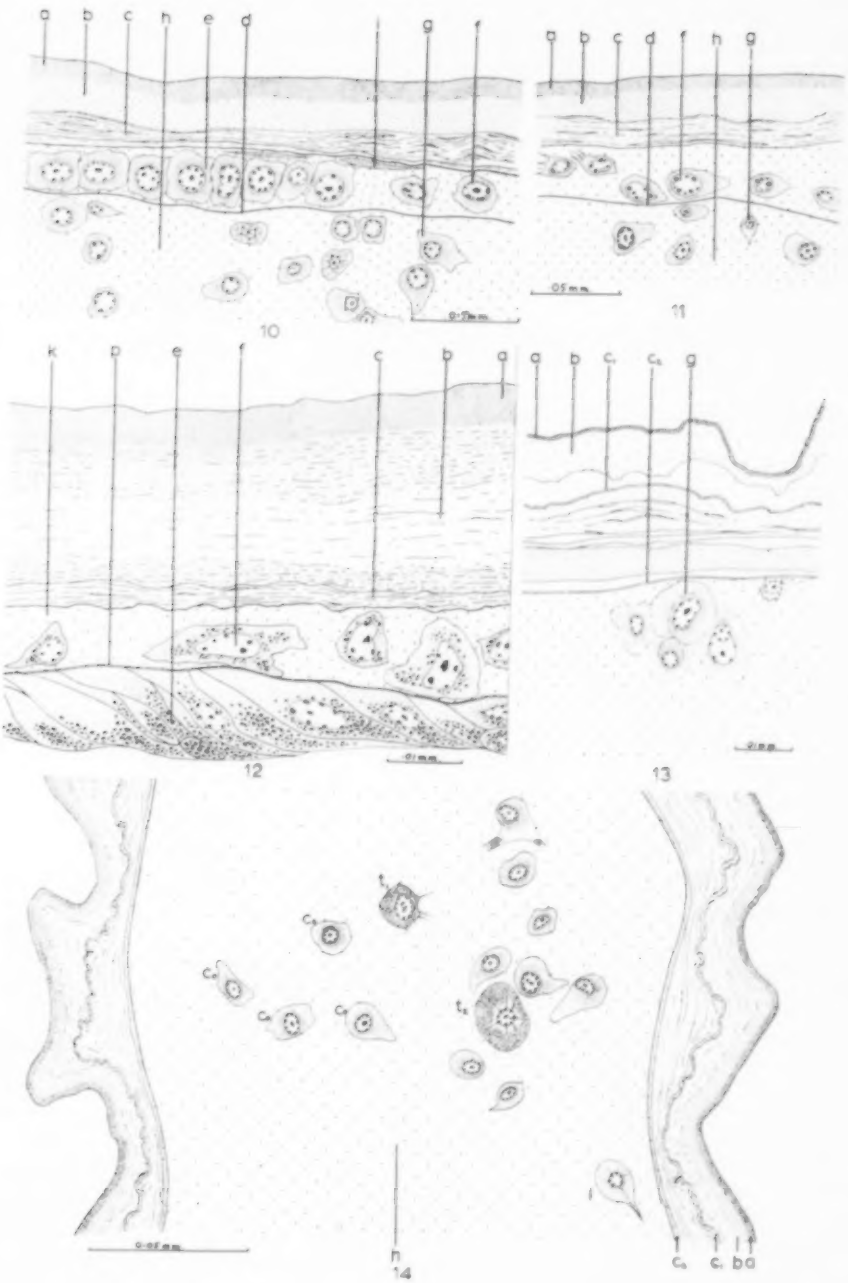
frequently walled off in this position, between layers of moulting membrane. This mostly occurs along the lateral sides of the gills, at the bases of the two rows of sensilla. The large tormogen cells of the latter are frequently left behind together with the adjacent epidermal cells. These epidermal cells are always rounded off but are frequently arranged in a unicellular layer. Only in one case have uncytolysed epidermal cells been identified in free suspension in the isolated fluid of the gills, and this was in the distal portion of the fourth segment. In all other gills they have been found walled off by the moulting membrane. Therefore, with the one exception these are epidermal cells separated from the sides of the retracting gill tissue early on in retraction. They have not been cut off by the retracting epidermal tip. However, in sections of fixed gills, dissociated epidermal cells are often indistinguishable from blood cells. It is possible therefore that free epidermal cells are overlooked in sectioned material.

The retraction of the epidermis in the terminal filament

Observations on the terminal filament showed that retraction of the epidermis begins 48 hr after the beginning of retraction in the gill filaments. In all cases tissue was left behind in the most distal portion of the filament. This tissue frequently becomes tanned and is a light amber colour. The retracting epidermis was observed to round off and retract rather like a deflating balloon. Where groups of fat cells were present within the filament, it was observed that these often led to a distortion of the retracting epidermis. In these cases, epidermal cells and fat cells were left behind within the isolated fluid of the terminal filament. Soon after being separated these appeared to be in a state of cytolysis. During retraction, the anus comes to lie in a posterior position, whereas in the larva it was dorsal. Most of the epidermis from the terminal filament comes to lie ventral and anterior to the anus after retraction. Ventrally, large paired white masses of proliferating cells can be observed which are the imaginal discs of the external copulatory organs. This new tissue replaces the majority of the larval pigmented epidermis in the eighth and ninth abdominal segments and the unpigmented epidermis retracted from the terminal filament. Epidermal cells are frequently left in the isolated fluid, especially in the larger female pharate pupae. There is much variation in the number left. Fat cells, blood cells, epidermal cells, and an oenocyte have been observed in the moulting fluid in the terminal filament. The oenocyte was already within the terminal filament before metamorphosis and was left behind in the fluid when the epidermis retracted.

Sections of the terminal filament showed much epidermal tissue left behind at the tip. Approximately 4 mm from the tip of the terminal filament of large female pupae, the epidermal cells round off and dissociate (Figs. 10, 11). The most posterior cells round off and dissociate first (Fig. 11). The basement membrane is clearly visible at this time in the terminal filament. The rounded off epidermal cells are almost indistinguishable from hyaline blood cells after fixation and staining, although the epidermal nuclei are usually larger and the cytoplasm more rounded and less granular. A few hours later, the basement membrane of the

VOL. 4 1960


a b c h e d g f
10
a b c d f h g
11
k p e f c b a
12
a b c c_4 g
13
c_4
c_3
c_2
c_1
t_1
t_2
h
14
c_4 c_3 c_2 c_1 ba

epidermis immediately behind that shown in Fig. 10 disappears, and the epidermal cells move inwards and unite to wall off the extreme tip of the terminal filament. Thus the isolated fluid in the extreme tip of the filament is not all secreted by the epidermis but some is haemolymph. This also occurs in the tips of the lateral gills. The isolated fluid contains both blood and dissociated epidermal cells. Once again it has not been possible to determine the fate of the epidermal cells left in the isolated fluid of the terminal filament. As the epidermis retracts after closing off the tip of the filament, it is highly susceptible to injury. There is no observable basement membrane, and the epidermal cells are rounded and moving inwards. Any slight increase in the blood pressure will break the continuity of the epidermal layer, freeing more blood into the isolated fluid. Likewise groups of fat cells or a stray oenocyte within the lumen of the terminal filament will cause a rupture of the epidermis. These breaks are quickly repaired, and the pharate pupae are not adversely affected. A frequent place of discontinuity of the epidermis is around the insertion of the dorsal and ventral longitudinal muscles of the ninth abdominal segment. These do not detach until 48 hr after the epidermis around has contracted away from the cuticle and they continue to function during this period. In the terminal filament the moulting membrane is not continuously secreted during retraction. It is secreted only when the epidermis is detaching from the larval cuticle.

VOL. 4 1960

Epidermal cells isolated in the wall of the abdomen

In the late pharate pupae 24 hr before ecdysis, pigmented cells can be seen floating within the moulting fluid, especially in the thorax. They swill around within the fluid each time the pharate pupa moves and show up against the light background of the unpigmented thoracic epidermis. These are pigmented epidermal cells from the abdomen. They are frequently isolated, especially in large female pharate pupae. The isolated cells are most commonly to be found in the dorsal and lateral posterior portions of the three terminal abdominal segments (Figs. 8, 12). The pigmented epidermal cells in sectioned material have highly irregular cytoplasmic boundaries. Unlike the blood cells, they are obviously moribund after isolation.

FIGS. 10, 11. Longitudinal section of the terminal filament at the beginning of tissue retraction. Fig. 11 is a section of the epidermis immediately posterior to that in Fig. 10.

FIG. 12. Pigmented epidermal cells isolated along the lateral wall of the abdomen at the larval–pupal moult.

FIG. 13. Partially clotted blood cells in the isolated fluid of the larval exuvea of the second gill segment, after pupal ecdysis.

FIG. 14. Blood cells fixed in the isolated fluid of the third segment of a gill. Cells t_1, t_2, are intermediate between hyaline and granular haemocytes. (Co) Coagulocyte hyaline haemocytes. (i) Cell intermediate between t_1 and Co.

(a) Exocuticle of larva. (b) Endocuticle of larva. (c) Highly laminated moulting membrane. (c_1) Outer layers of moulting membrane. (c_2) Inner layers of moulting membrane. (d) Basement membrane. (e) Epidermal cells. (f) Dissociated epidermal cells. (g) Blood cells. (h) Haemolymph. (i) Nerves obliquely sectioned. (k) Moulting fluid. (p) Pupal cuticle.

Cells found within the larval exuvia after the larval–pupal ecdysis

Sectioned and stained exuvia, heat fixed immediately after ecdysis, showed the presence of many cells. These are epidermal and blood cells. The latter (Fig. 13) were invariably in a semi-clotted state and usually clumped together. It seems that the majority of blood cells clot at ecdysis in the isolated fluid unabsorbed within the gills. Many epidermal cells are also found, always walled off from the lumen of the gills by layers of moulting membrane. On one occasion only were epidermal cells found walled off within the lumen of the gill in the distal portion of segment 4. The epidermal cells walled off by a moulting membrane are invariably rounded but have clearly discernible cell walls. This is very different from epidermal cells isolated in the lateral walls of the abdomen (Fig. 12). The latter cells are moribund and the cell boundaries are difficult to discern.

Nerves are frequently found in the exuvia after ecdysis, particularly those of the two large terminal sensilla of each gill. Nerves together with epidermal cells are also frequently found walled off by moulting membrane along the lateral walls of the gills.

Wound repair by isolated gill tissue

After the gill tips of pharate pupae had been cut off 18 hr before ecdysis, tanned wound plugs formed across the open ends of the larval cuticle. If the removed tips were left in the air without extruding the isolated fluid, tanned wound plugs formed across the open ends. Pin pricks made in the gills were repaired by tanned wound plugs. The plugs darkened in 1 hr to a dark amber to black colour. In final instar larval gills, wound plugs darken to an intense black after only 25 min. Tanned plugs 24 hr old in the gills of late pharate pupae did not disperse in cold concentrated nitric or hydrochloric acid. They withstand hot concentrated nitric acid for more than 5 min. If the the plugs are of sclerotin the presence of a tryosinase inhibitor should prevent the plug from becoming darkened. A solution of 0·5 N phenyl-thiourea in 1 per cent ethyl alcohol completely inhibited the formation of tanned plugs. Two gill tips were cut off and placed in 1 per cent ethyl alcohol with no phenyl thiourea, to check the toxicity of the ethyl alcohol. In both tanned plugs were formed. Therefore 1 per cent ethyl alcohol does not prevent tanning. The wound plugs are of tanned protein or sclerotin. Care has to be exercised in wounding the gills, for it is possible to burst open the retracting tip of the epidermis when piercing the cuticle. In the detached gill tips, the pupal epidermis or blood can have played no part in the formation of the wound plugs.

In late pharate pupae and early emerged pupae the haemolymph, unlike the isolated fluid, shows very little clotting behaviour. Emerged pupae or late pharate pupae, with punctured pupal body walls, usually bleed to death. The lack of clotting is because there are few coagulocyte, hyaline haemocytes, and much lipoid material present in the haemolymph. The lipoid material reduces the ability of blood to form a clot.

Each day a gill was cut off and the isolated fluid sampled from six pharate pupae. It was found that clotting decreased with time. On the seventh day, immediately before reabsorption of the moulting fluid before ecdysis, very little clotting was observed. The degree of the tanning of the wound plugs also declined with time. On the seventh day there was little or no tanning.

Thus the formation of clots in wounds in the larval cuticle of the pharate pupa is in marked contrast to the lack of clotting in wounds in the pupal cuticle.

DISCUSSION

Blood cells, epidermal cells, fat cells, and an oenocyte, have been identified at the larval–pupal moults within the isolated fluid of *Sialis lutaria*. The number and type of cells isolated is variable. It seems to be dependent upon mechanical factors. Generally speaking, the larger the pharate pupa, the greater the density of cells left behind in the isolated fluid. It appears that only blood-cells remain viable within the isolated fluid, other cells quickly break down. If epidermal cells remain viable in the isolated fluid of the terminal filaments, they are completely dissociated and indistinguishable structurally and in their behaviour from hyaline haemocytes. This is possible though the majority of the epidermal cells in the gills can be seen to cytolyse at the retracting gill tip.

VOL. 4 1960

Epidermal cells are isolated between the lamellae of the moulting membrane in the gills and in the isolated fluid between the pupal cuticle and the moulting membrane along the lateral body wall of the abdomen. Because they are pigmented they are the only identifiable cells in the isolated fluid. The limits of their cytoplasm tend to be irregular and many are completely cytolysed, the pigment granules being found free in the isolated fluid. No identifiable epidermal cell in the isolated fluid was other than moribund.

The greatest number of cells are cut off in regions where there is the greatest reduction in the surface area at the larval–pupal moult. Large tissues, like groups of fat cells, are not normally present within appendages lost during development. Where they are accidentally present they add considerably to the difficulty of epidermal retraction. The insect is normally unable to retract these tissues and they are isolated together with the surrounding epidermal cells. This results in a breach in the epidermis. Therefore the haemolymph is temporarily in continuity with the isolated fluid. These discontinuities in the epidermis are quickly repaired as retraction continues. All discontinuities in the epidermis occur in the early pharate pupa before the secretion of the pupal cuticle. Discontinuities do not occur in the epidermis of the first seven abdominal segments except in the lateral gills. However, a few epidermal cells are left in the isolated fluid of the first seven abdominal segments, although their number is small.

No cells of any type have been identified in the isolated fluid of the pupal–adult moult of *Sialis lutaria*. This is in marked contrast to *Lipsothrix* and *Taphrophila* (HINTON, 1955, 1957), where epidermal cells and haemolymph, but not blood cells were isolated at the pupal–adult moult. In these the structures in which the cells were isolated were pupal. The epidermal cells in the gills of *Sialis lutaria*,

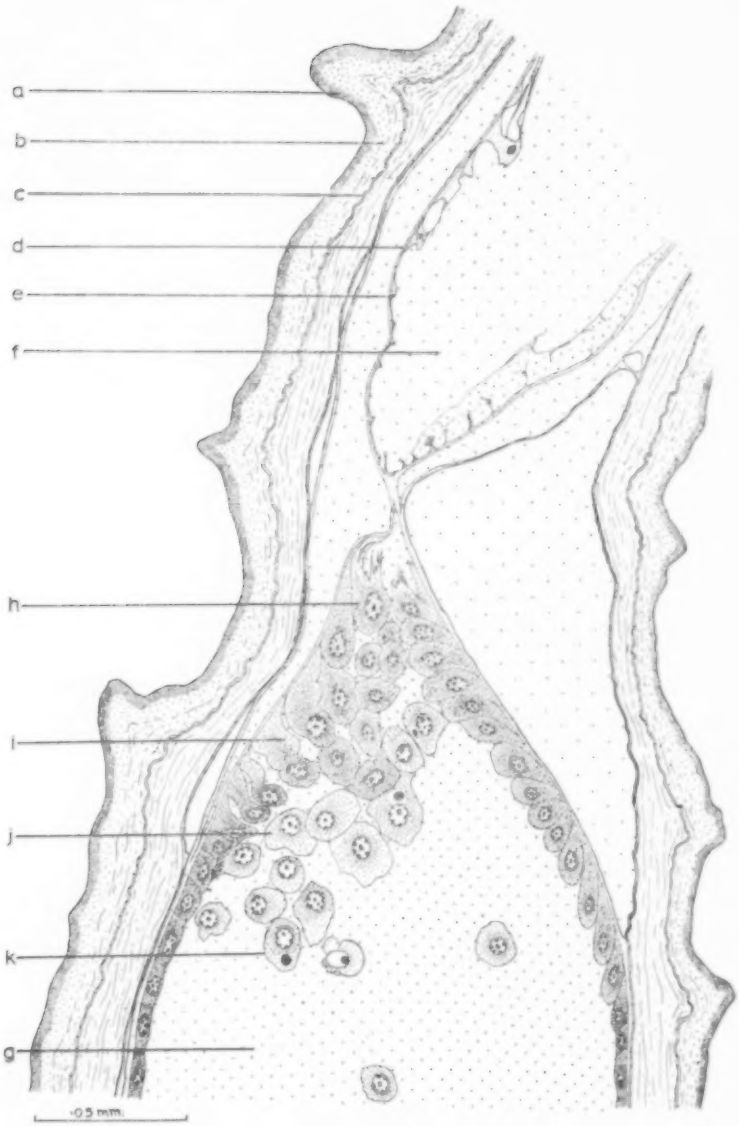


FIG. 15. The retracting epidermis in the third gill segment. (a) Exocuticle of larva. (b) Endocuticle of larva. (c) Outer lamellae of moulting membrane. (d) Inner lamellae of moulting membrane. (e) Remains of cytolysed epidermal cells. (f) Isolated fluid. (g) Haemolymph. (h) Dissociating epidermal cells. (i) Rounding off and elongating epidermal cells. (j) Blood cells. (k) Blood cells with ingested particles.

unlike those of *Taphrophila vitripennis* (HINTON, 1957), quickly cytolyse leaving only blood cells. The latter remain fully viable within the isolated fluid of *Sialis lutaria*. Their activity slowly declines and they become moribund in the activated fluid. In this period, except for the last 24 hr before ecdysis, the blood cells are able to repair wounds in the larval cuticle. In the late pharate pupal stage the capacity of the blood of the pharate pupa to clot is much reduced or lost. There is a slight variation in the capacity to clot which is related to the time of the disintegration of the fat body. Thus blood cells in the isolated fluid appear to be of advantage to the insect. The number of epidermal cells present in the moulting fluid of *Sialis* gills is doubtful because of the possibility of their confusion with blood cells. The difficulties of fixation, together with the degree of clotting exhibited by the cells, indicates that the vast majority must be blood cells of the coagulocyte, hyaline haemocyte, type.

PASSONNEAU and WILLIAMS (1953) described the moulting fluid of Cecropia silkworm pupae as a gel turning to a sol upon activation. The isolated fluid of *Sialis* is at all times a sol. Up to the time of its activation the isolated fluid of *Sialis* will begin to clot when the larval cuticle is punctured. It is conceivable that both have a similar protective function. PASSONNEAU and WILLIAMS (1953) found that the early moulting fluid of Cecropia was of a similar chemical composition qualitatively, if not quantitatively, to blood. This was not true for the late moulting fluid. The quantitative differences between early moulting fluid and blood might be accounted for by the presence of cellular elements in the haemolymph. All the blood cells cannot possibly be removed from haemolymph at any stage in an insect, for the coagulocytes will break down and discharge cellular material into it. PASSONNEAU and WILLIAMS observed no cells in the moulting fluid at the pupal–adult moult of Cecropia, nor were any observed by HINTON (1958) in the moulting fluid of *Simulium*. Thus it is possible that early inactive moulting fluid could be the same or very nearly the same as pure haemolymph. This would agree with observations on *Sialis* where blood cells appear quite normal in isolated fluid and where moulting fluid and haemolymph are known to mix. Moreover, no dermal glands have ever been observed in the epidermis of *Sialis*. Therefore the moulting fluid must be secreted by or pass through the epidermis as a whole. It must be noted that the oenocytes are in a highly active state in the late pharate pupal stage and could well be responsible for the production of an activating agent of the moulting fluid.

During the pharate pupal stage, the pupa is enveloped by the old larval cuticle. During most of this time, the pupa, which during a cold snap in the field may last a few weeks, is not completely enveloped in pupal cuticle. The retracted gills, terminal filament, and ventral surface of the last two abdominal segments do not secrete a pupal cuticle until the very late pupal stage. It is possibly significant that the maximum thickness of the larval cuticle and moulting membrane in the gill is almost equal to the total thickness of the final larval instar cuticle and moulting membrane in the abdomen, although the thickness of the larval cuticles are very different. In the terminal filament where the original final instar larval cuticle is

VOL. 4 1960

thick, only a thin moulting membrane is secreted at the time of detachment of the epidermis, and here there is no continuous secretion of moulting membrane by the retracting epidermis.

In specimens where the duration of retraction has been lengthened by lowering the temperature, there is no detectable increase in the thickness of the moulting membrane as a result of the treatment. Most of the endocuticle of the final instar larva, and also the moulting membrane secreted at the larva–pupal moult, is dissolved immediately before ecdysis, but only where it is in proximity to the body of the pupa. In the walled-off gills the endocuticle is not re-absorbed. Therefore the activation of the moulting fluid only occurs where it is in close proximity to the epidermis of the pupa. The activation of the moulting fluid is local. This agrees with the observation of WILLIAMS (1952) on moulting fluid activation in Cecropia silk moths. He found that where areas of pupal cuticle were induced to undergo a second moult, the endocuticle was dissolved, but not in other areas of the same insect.

The secretion of moulting membrane during retraction may slightly reduce the danger of dehydration. Any pharate pupa removed from its cell and exposed to the open air will in a few hours lose sufficient water to be unable to split open the larval cuticle at ecdysis. The cuticle newly secreted at moulting seems to have little functional significance except that of affording some degree of protection to the retreating gill tip. It is conceivable that the thick moulting membrane within the gills may serve to strengthen the larval cuticle during epidermal retraction and therefore prevent the thin-walled gills folding over with consequent loss of large amounts of cellular material. Only the inner part of the new cuticular secretion, that secreted immediately before the secretion of the pupal cuticle, is considered to be equivalent to the moulting membrane of other insects. That is, the thin hardened inner lamellae not dissolved when exposed to activated moulting fluid. The observations on the moulting membrane of *Sialis* are not inconsistent with those of RICHARDS (1955), PASSONNEAU and WILLIAMS (1953), and MALEK (1958). The staining reaction in all four are identical. MALEK (1958) considers the moulting membrane of the adult *Schistocerca* to be the innermost layers of the endocuticle of the larva. These latter layers are resistant to moulting fluid. After activation of the moulting fluid, they are left as a discrete membrane. *Sialis* differs in the extreme thickness of the newly secreted cuticle especially within the gills. *Sialis* provides additional evidence that the moulting membrane is secreted immediately before moulting (RICHARDS, 1955). It is of interest that MALEK (1958) noted that the moulting membrane is reduced by the action of the moulting fluid, as was found in *Sialis*. Moreover, after the activation of the moulting fluid, the thickness of the moulting membrane of the abdomen of *Sialis* is comparable to that of *Schistocerca*, and both are multi-layered. In sections and in whole gills of *Sialis lutaria*, OCHSÉ (1944) did not observe the presence of cells in the moulting fluid or the secretion of the moulting membrane during the moult. He noticed a wide variation in the lamination of the endocuticle in the gills. This was at a time before the activation of the moulting fluid. In a diagram he drew the larval

endocuticle much thicker after the retraction of the epidermis than before retraction, but does not mention this in his text. It is possible that using chemical fixatives warmed to only 40°C, OCHSÉ did not fix the tissue quickly enough to show blood cells in the moulting fluid.

Acknowledgement—My best thanks are due to Dr. H. E. HINTON for his criticism and advice during the course of this work.

REFERENCES

BURSELL E. (1958) The water balance of tsetse pupae. *Phil. Trans.* (B) **241**, 179–210.

GEIGY R. (1948) Etude expérimentale de la métamorphose de *Sialis lutaria* L. *Bull. biol.* **33**, 62–67.

GEIGY R. and OCHSÉ W. (1940a) Schnürungsversuche an larven von *Sialis lutaria* L. *Rev. suisse Zool.* **47**, 193–194.

GEIGY R. and OCHSÉ W. (1940b) Versuche uber die inneven Faktoren der Verpuppung bei *Sialis lutaria* L. *Bull. biol.* **47**, 225–241.

GEIGY R. and RAHM U. H. (1951) Beiträge zur experimentellen Analyse der Metamorphose von *Sialis lutaria* L. *Bull. biol.* **58**, 408–413.

HINTON H. E. (1955) The structure of the spiracular gill of the genus *Lipsothrix* (Tipulidae) with some observations on the living epithelium isolated in the gill at the pupa–adult moult. *Proc. R. ent. Soc. Lond.* (A) **30**, 1–14.

HINTON H. E. (1957) The structure and function of the spiracular gill of the fly *Taphrophila vitripennis*. *Proc. roy. Soc.* (B) **147**, 90–120.

HINTON H. E. (1958) Concealed phases in the metamorphosis of insects. *Sci. Progr.* **46**, 260–275.

KÜHN A. and PIEPHO H. (1938) Die Reaktionen der Hypodermis und der Versonschen Drüsen auf das Verpuppungshormon bei *Ephestia kühniella* Z. *Biol. Zbl.* **58**, 12–51.

MALEK S. R. A. (1958) The origin and nature of the ecdysial membrane in *Schistocerca gregaria* (Forskål). *J. Ins. Physiol.* **2**, 298–312.

OCHSÉ W. (1944) Experimentellen und Histologische Beiträge sur inneren Metamorphose von *Sialia lutaria* L. *Rev. suisse Zool.* **51**, 1–82.

PASSONNEAU J. V. and WILLIAMS C. M. (1953) The moulting fluid of the Cecropia silkworm. *J. exp. Biol.* **30**, 545–560.

RAHM V. H. (1952) Die innersekretorische Steuerung der postembryonalen Entwicklung von *Sialis lutaria* L. *Rev. suisse Zool.* **59**, 179–237.

RICHARDS A. G. (1952) Studies on arthropod cuticle—VIII. The antennal cuticle of honey-bees with particular reference to the sense plates. *Biol. Bull., Woods Hole* **103**, 201–225.

RICHARDS A. G. (1955) Studies on arthropod cuticle—XI. The ecdysial membrane. *J. Morph.* **96**, 537–563.

WILLIAMS C. M. (1952) Morphogenesis and the metamorphosis of insects. *Harvey Lect.* **47**, 126–155.

VOL. 4 1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 258 *to* 269. *Pergamon Press Ltd., London. Printed in Great Britain*

BIOCHEMICAL STUDIES ON INSECT HAEMOLYMPH—I. VARIATIONS IN REDUCING POWER WITH AGE AND THE EFFECT OF DIET

G. F. HOWDEN and B. A. KILBY

Imperial College Field Station, Ashurst Lodge, Sunninghill, Ascot, Berks., and Department of Biochemistry, University of Leeds

(*Received* 19 *December* 1959)

Abstract—The variation with age of eight reducing values of the haemolymph of *Schistocerca gregaria* was determined both for locusts reared entirely on grass and for those reared on an artificial diet to which a very small quantity of grass had been added. Random variation in results found in the former group was considerably reduced by the use of artificial diet, although in each case the normal patterns of variation with age were quite clear. Measurements of the specific gravity of the haemolymph of *S. gregaria*, together with LEE's values for the variation in blood volume with age, and the above reducing values, have enabled a calculation to be made of the variation with age of the total amounts of various reducing materials present in locust haemolymph. In particular, a very considerable increase in the trehalose content and a much smaller increase in glucose were shown to occur during the fifth instar.

INTRODUCTION

THE reducing power of insect blood, expressed as mg glucose per 100 g haemolymph, has been shown by FLORKIN (1937) and KUWANA (1937) to vary in a regular way with age in holometabolous insects. The purpose of the work reported here was to study the same phenomenon in a heterometabolous insect in somewhat greater detail; to investigate further, firstly, the observation made by both FLORKIN (1937) and by KUWANA (1937), that a large part of the total reducing power of insect blood is due to the presence of non-fermentable reducing substances, and secondly, the observation of KUWANA (1937) that the reducing power of haemolymph is greatly increased by acid hydrolysis. Investigations were begun on the desert locust *Schistocerca gregaria* Forskål and as they progressed it became apparent that variations in diet were affecting results. For this reason an artificial diet, supplemented by very small quantities of grass, was substituted for the all-grass diet (HOWDEN and HUNTER-JONES, 1958). This considerably reduced the random variation and greatly clarified the patterns of variation of reducing powers with age.

MATERIALS

The locusts used were reared from stocks supplied by the Anti-Locust Research Centre. The method of rearing was as described by HUNTER-JONES (1956) with the following modifications:

(1) *Lighting*. This was provided by one 75 W bulb set in the roof of each cage and controlled by a Venner time switch set to give 16 hr light and 8 hr darkness.

(2) *Temperature*. This was kept at about 35°C during the day but fell to 28°C at night in the rearing cages. However, in the thermostatically controlled experimental cage, in which marked locusts were placed, the temperature only varied between 35 and 37°C.

(3) *Water*. This was supplied to all cages in Petri dishes containing cotton wool.

(4) *Relative humidity*. This varied from a probable 100 per cent on the grass and Petri dishes to 30 per cent in the rest of the cage.

(5) *Food*. Two sets of values of reducing powers, referred to as 'winter batch' and 'summer batch' results, were obtained by feeding the locusts on, respectively, grass alone during the winter; and the artificial grassmeal diet together with very small daily additions of grass during the summer.

METHODS

Marking. In order that results could be obtained from insects of known age, all locusts were marked on the day of their ecdysis into the fifth instar and placed in the experimental cage. More accurate estimates of age near the end of the fifth instar were made on the basis of posture and coloration.

VOL. 4 1960

Extraction and deproteinization of haemolymph

Blood from at least five locusts per estimation was taken from the thoracic haemocoel by means of a fine glass capillary and deproteinized by the addition of 8·0 ml N/12 sulphuric acid and 1·0 ml 10 per cent sodium tungstate for 0·5 g haemolymph (and *pro rata* for larger quantities). After stirring, the mixture was left on ice for 10 min to complete precipitation and then centrifuged (3000 g) for 10 min. BERKMAN (1954) has shown that this procedure removes all protein but not all peptides. The supernatant was removed and the centrifugate washed twice with distilled water. The combined supernatants and washings were made up to 25·0 ml. Eight reducing values were obtained from each 25·0 ml deproteinized haemolymph.

Estimation of reducing power

Each result was based on duplicate determinations on 2·0 ml aliquots of deproteinized haemolymph. Estimations of reducing power were made by a modification of the Hagedorn and Jensen method (HAWKE, OSER, and SOMERSON, 1947). This method of estimation was used both because it is accurate and because it was used by KUWANA (1937) and thus allowed direct comparison of results. It involved a heating stage the duration and temperature of which affected the results. Using a glucose solution of known concentration it was found that if the aliquot was heated with 0·005 N potassium ferricyanide in a pressure cooker at 15 lb/in^2 pressure for 15 min the correct result was obtained. These results are referred to as 'hot values'. Omission of this heating stage gave 'cold values'.

EXPERIMENTAL PROCEDURE

Duplicate determinations on aliquots of the pooled deproteinized haemolymph were made to obtain the following values:

(1) *Cold values.* The reducing power of unhydrolysed haemolymph measured by the above method but with the omission of the heating stage.

(2) *Cold hydrolysed values.* Two millilitre aliquots were hydrolysed by the addition to each of 1·0 ml N sulphuric acid and subsequent heating at 15 lb/in² for 30 min in a pressure cooker. The solution was then neutralized and the reducing power estimated as for cold values.

(3) *Hot hydrolysed values.* The reducing power of haemolymph after acid hydrolysis. The aliquot being hydrolysed as for cold hydrolysed values, neutralized and estimated for reducing power by the standard method.

(4) *Total reducing power.* The reducing power of unhydrolysed haemolymph measured by the standard method.

(5) *Non-fermentable values.* The reducing power of unhydrolysed haemolymph after fermentation by baker's yeast. Baker's yeast was pre-treated for the fermentation by suspending 6 g fresh yeast in 50 ml distilled water for 1 hr. The suspension was then centrifuged and the residue washed five times by resuspension and centrifugation. The final residue was suspended in 35 ml distilled water. Two millilitre aliquots of haemolymph were incubated with 5·0 ml of the yeast suspension in small centrifuge tubes at 32°C for 30 min. The suspension was then centrifuged, the supernatant removed, and the residue washed twice with distilled water. The combined supernatant and washings were estimated for reducing power by the standard method. Blank estimations were carried out on 2·0 ml aliquots of a solution obtained by the above procedure from the incubation of yeast with distilled water.

(6) *Hydrolysed non-ferm entablevalues.* The reducing power of hydrolysed haemolymph after fermentation by baker's yeast. Aliquots containing 1·0 ml haemolymph solution + 1·0 ml distilled water hydrolysed by the addition to each of 1·0 ml N sulphuric acid as above. The neutralized solutions were then fermented and the reducing power measured as described for the non-fermentable values. Blank determinations were made by fermenting aliquots containing 1·0 ml N sodium sulphate plus distilled water.

The amounts of true blood sugar and 'polysaccharide' were obtained by calculation from the above values.

(7) *Fermentable or true blood sugar.* This was obtained by subtraction of (5) from (4).

(8) '*Polysaccharide*'. Obtained by subtracting the sum of (6) and (7) from (3). This value represented those substances which, after acid hydrolysis, produced substances fermentable by baker's yeast. Trehalose was subsequently shown by Howden and Kilby (1956) to constitute the major part of this value.

RESULTS

Most of the analyses were carried out on female adults or 'pre-female' fifth-instar larvae as the amount of haemolymph obtainable from these was considerably greater than from males. The variations with age of each value are shown in Figs. 1–8.

This method of estimating reducing power together with the above procedures, carried out on solutions of glucose and trehalose of known concentration indicated that the experimental error was within ± 5 per cent. The age represented by the points on the graphs was estimated from the time of the previous moult and was usually known to within ± 6 hr, but occasionally only to within ± 12 hr.

For most values the graphs of both 'winter' and 'summer' batch locusts are of the same shape but the latter values tend to be higher. This difference varies between 75 and 100 mg for cold values, cold hydrolysed values, total reducing power, and non-fermentable values, the graphs of which all have a flattened U-shape. There is no difference for true blood sugar between 'winter' batch and 'summer' batch locusts. The difference between these batches for values involving 'polysaccharide' is, however, very much greater, being about 500 mg/100 g.

VOL. 4 1960

Comparison of 'winter' and 'summer' batch results shows that the scatter of results in the 'winter' batch series was considerably reduced by feeding the locusts on artificial diet together with small additions of grass, and that in order to compare quantitative biochemical data from locusts of different ages it is essential to feed them on a constant diet besides making all other rearing conditions uniform.

Specific gravity. Some measurements of the density and specific gravity of the haemolymph of *S. gregaria* were made in order to calculate, from the above values of reducing power and LEE's (1958) figures for the variation in blood volume with age, the variation with age of the *total* amounts of reducing materials in the haemolymph of *S. gregaria*.

Measurements were made on haemolymph taken from the 'pre-male' and 'pre-female' fifth-instar larvae of known age, fed during this stadium entirely on the artificial grassmeal diet plus water. The mean value of the density was found to be 1·0377 g/ml at 21°C. No significant variation with age was found, nor was there a significant difference between the sexes, though the variation in results as between batches of individuals was ± 1 per cent. The specific gravity therefore averaged 1·0397.

DISCUSSION

The results given in Figs. 1–8 show that the patterns of variation with age of total reducing power and of the cold values of the haemolymph of fifth-instar 'pre-female' *S. gregaria*—the heterometabolous desert locust, are similar in shape to those found for the holometabolous silkworm *B. mori* by KUWANA (1937), though the actual values were somewhat higher in the desert locust than in the silkworm.

They also reveal an accumulation of glucose from 0–5 mg/100 g at the beginning of the fifth instar, to about 130 mg/100 g at the 108th hr, the latter concentration

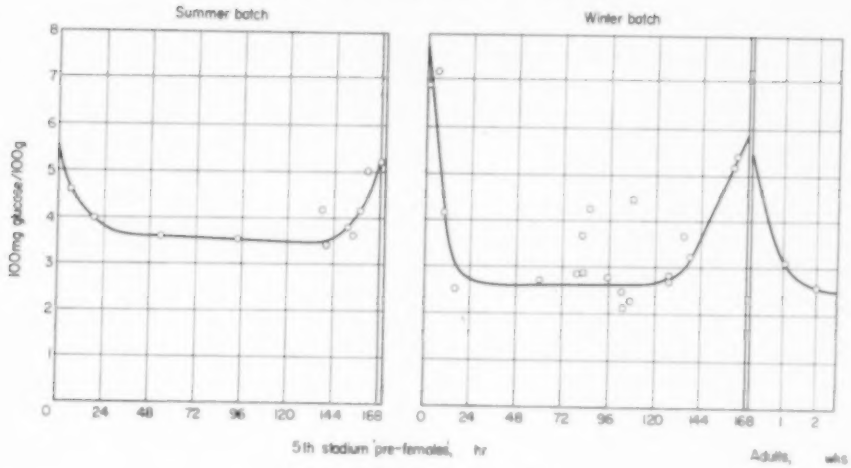

Summer batch
Winter batch
100mg glucose/100g
0 24 48 72 96 120 144 168
0 24 48 72 96 120 144 168 1 2
5th stadium 'pre-females', hr
Adults, wks

FIG. 1. Total reducing power.

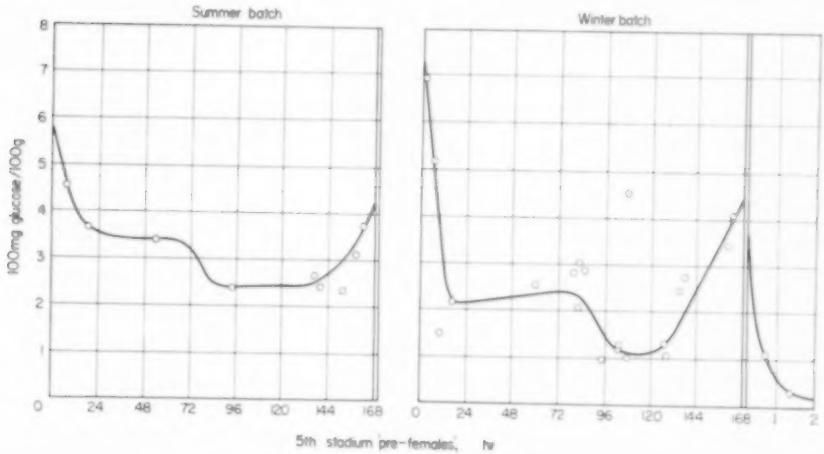


FIG. 2. Non-fermentable values.

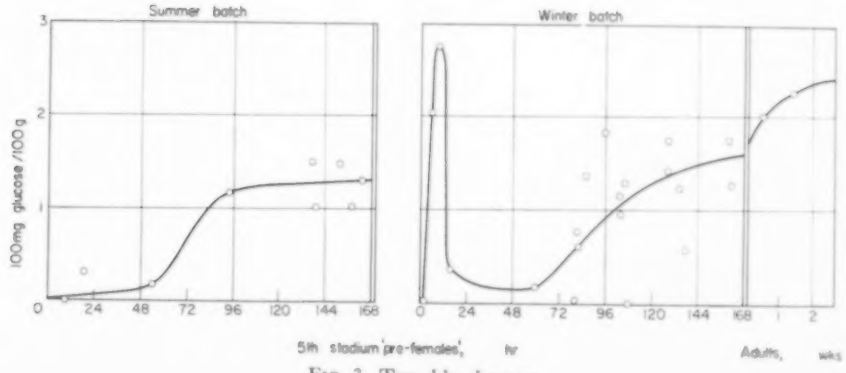

Summer batch
Winter batch
100mg glucose/100g
0 24 48 72 96 120 144 168
0 24 48 72 96 120 144 168 1 2
5th stadium 'pre-females', hr
Adults, wks

FIG. 3. True blood sugar.

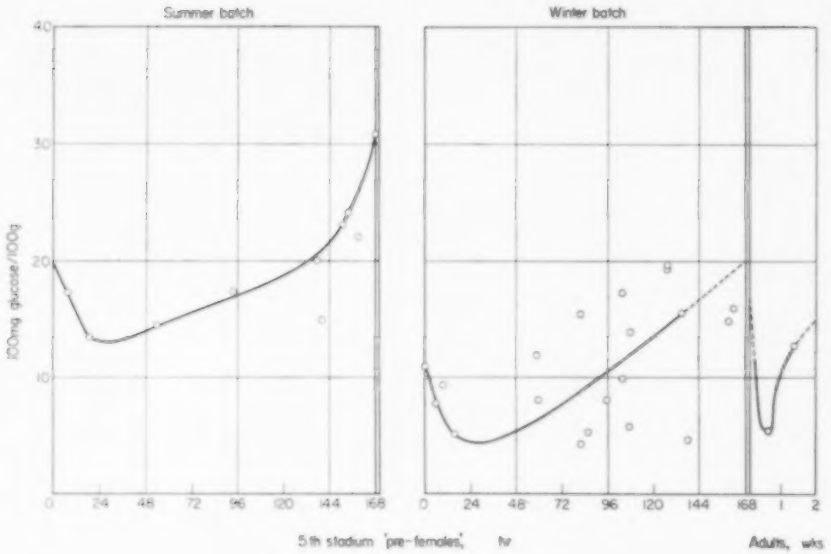


FIG. 4. Hot hydrolysed values.

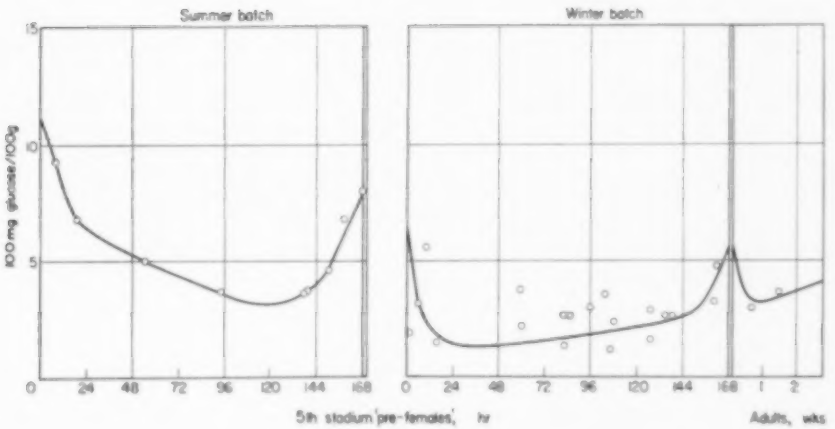


FIG. 5. Hydrolysed non-fermentable values.

VOL. 4 1960

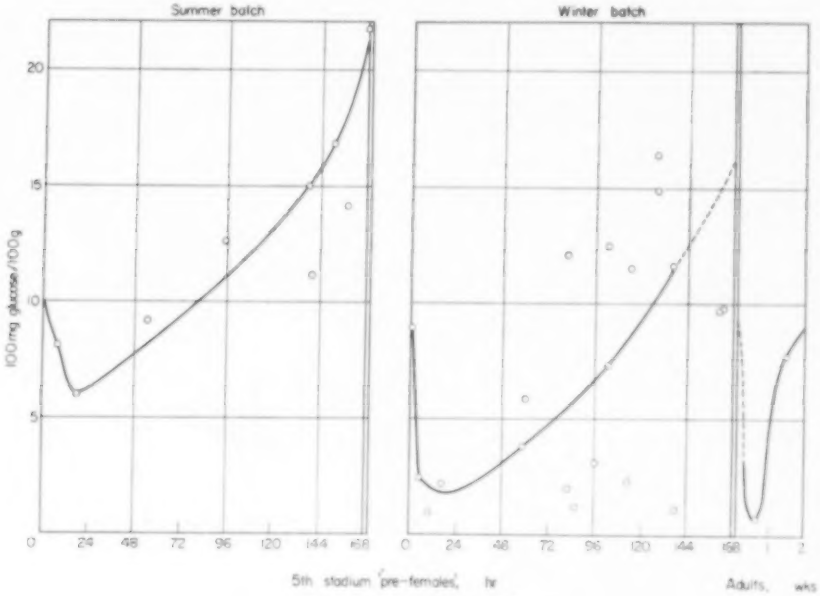


Fig. 6. 'Polysaccharide'.

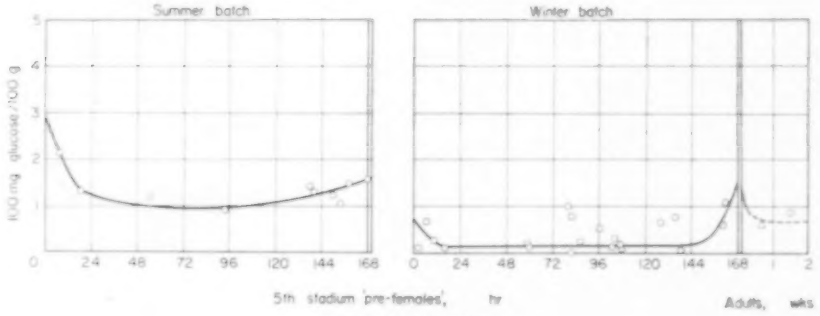


Fig. 7. Cold values.

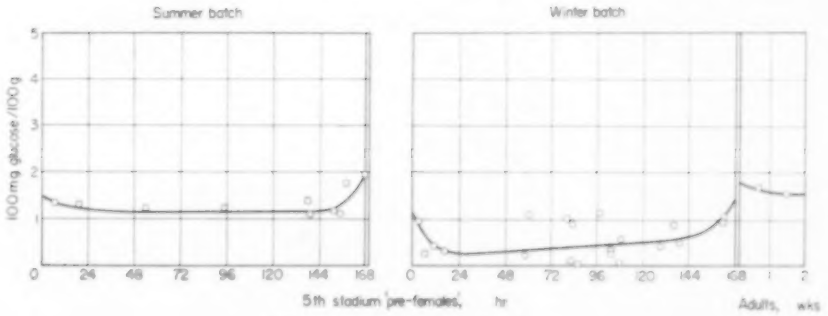


Fig. 8. Cold hydrolysed values.

Figs. 1–8 showing the variation with age of reducing values of the haemolymph of *S. gregaria*.

being maintained until the end of the instar. The hot hydrolysed values and the 'polysaccharide' concentration vary with age in an entirely different manner, falling rapidly in the first 18 hr, and then rising during the rest of the fifth instar. Thus the concentration of 'polysaccharide' in the summer batch locusts rose to just over 2000 mg/100 g at the end of the instar.

This 'polysaccharide' fraction has been shown to consist very largely of trehalose (HOWDEN and KILBY, 1956). TREHERNE (1958a) has also measured the concentrations of glucose and trehalose in adult female desert locusts and found average values of 24·1 mg/100 g for glucose and 694·5 mg/100 g for trehalose. However, an accurate comparison of the two sets of figures is not possible both because TREHERNE has not specified the age of his locusts and because they were reared on maize. Nevertheless the two sets of results are of the same order of magnitude.

Table 1 shows a comparison of the reducing values of 'pre-male' and 'pre-female' locusts of the same age fed on grass. It may be noted that the only large differences between the sexes is in those figures reflecting the trehalose content of the haemolymph.

VOL. 4 1960

TABLE 1—A COMPARISON BETWEEN THE REDUCING VALUES OF THE HAEMOLYMPH OF 'PRE-MALE' AND 'PRE-FEMALE' FIFTH INSTAR *S. gregaria* OF THE SAME AGE

Reducing values mg/100 g	'Pre-males' 106 hr	'Pre-females' 106 hr
Cold value	15	20
Cold hydrolysed value	9	30
Total reducing power	228	275
Non-fermentable value	98	130
True blood sugar	130	125
Hot hydrolysed value	584	1400
Hydrolysed non-fermentable value	128	220
'Polysaccharide'	326	1055

LEE (1958) has more recently shown that there is a straight line increase in total blood volume of fifth-instar *S. gregaria* females fed entirely on the artificial grassmeal diet plus water, from 100 μl. at the beginning of this stadium to 360 μl. at the end.

These figures taken together with the measurements of density and the figures for concentration of reducing materials have enabled a calculation to be made of the *total* amounts of the various reducing materials.

In Figs. 9–13 can be seen the results of these calculations for the variation with age of the following—'total reducing material' (from T.R.P.), trehalose (from 'polysaccharide'), glucose (from T.B.S.), 'non-fermentable reducing substances' (from non-fermentable values) and 'cold reducing substances' (from cold values). Since LEE's locusts were fed on artificial diet and those used for estimation of

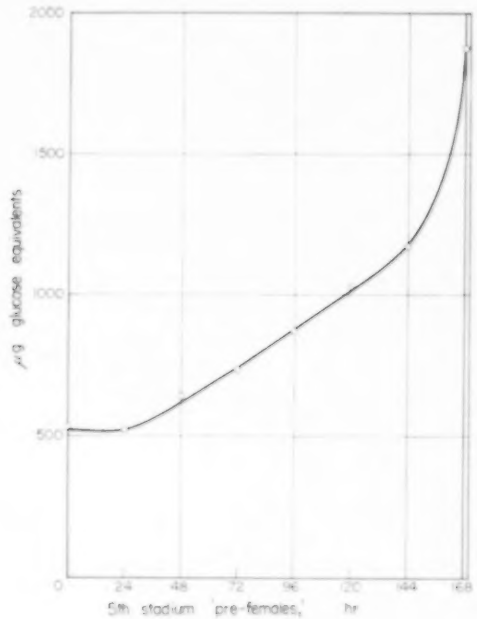


FIG. 9. Total reducing material.

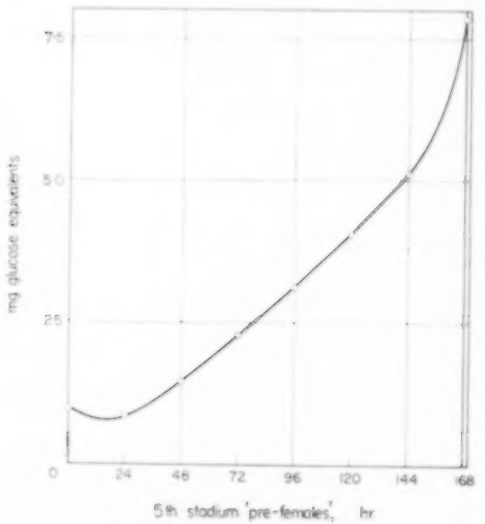


FIG. 10. Total trehalose content.

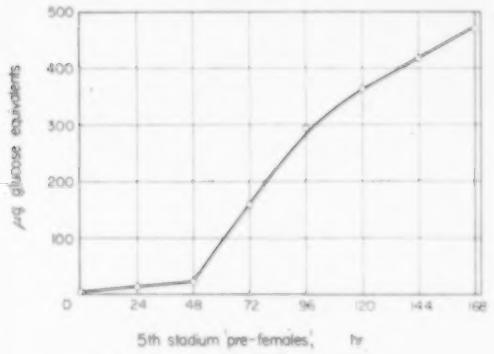


FIG. 11. Total glucose content.

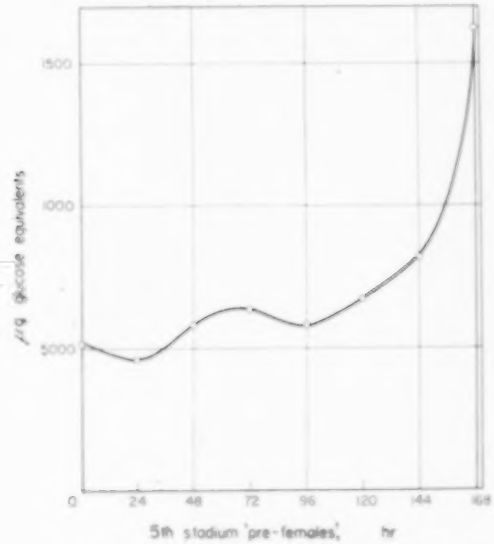


FIG. 12. Non-fermentable reducing material.

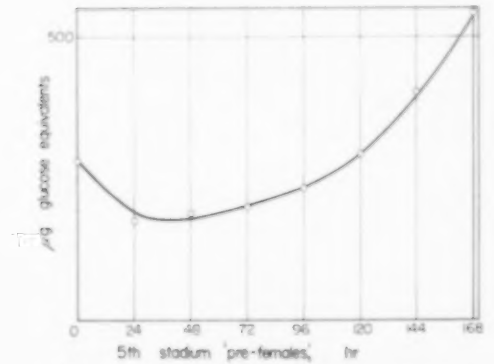


FIG. 13. Total cold value reducing material.

FIGS. 9–13 showing the variation with age in the total amounts of reducing materials in the haemolymph of fifth-instar 'pre-female' *S. gregaria*. The values shown represent the total amount of reducing material in the haemolymph of an individual insect at a given age.

reducing values were fed small quantities of grass in addition to this diet, the figures for total quantities cannot be exact. However, interesting observations can be made from these graphs which accord well with the fact that *S. gregaria* does not eat for 24 hr after the moult into the fifth instar. In these first 24 hr trehalose is broken down to the extent of about 200 μg, some of which is used to increase the total true blood sugar from 5 to 14 μg. The rest is presumably used as the major source of energy, an active trehalase being present in both haemolymph and fat body (Howden and Kilby, 1956). Subsequently it was found that the trehalose content of adult *S. gregaria* haemolymph fell after 1 hr flying by an average of 730 mg/100 g haemolymph. Confirmation of this suggestion, that trehalose is a major source of energy for flight, was provided by the observations of Bücher and Klingenberg (1958), who found that the decrease in trehalose content of *L. migratoria* flight muscle after 120 min flight amounted to 167 μM/g fresh weight flight muscle.

Also during the first 24 hr of the fifth instar there is a decrease in the total amounts of non-fermentable and of cold value reducing material of the order of 60 and 100 μg respectively. If such material is used in the build-up of body tissue such a fall is to be expected during the period when the cuticle is hardening and darkening. The similarity in the shape of the patterns of variation with age of the non-fermentable values and cold values suggests the possibility that they are both due to the same group of substances, the former values being due to reaction with ferricyanide with heat and the latter either this reaction without heat or a simple reaction with iodine.

During the rest of the instar there is an increase in the total amounts of all the reducing substances and of trehalose. However, the fact that the locust does not eat under these conditions during the last 24 hr of this instar indicates, since the total amounts of reducing materials continue to rise during this period, that these materials are produced during the final 24 hr by the breakdown of other substances either in the haemolymph or in other tissues. In particular, the amount of trehalose increases more during the last 24 hr than in the preceding 24 hr period. This can only be due to its production from other substances. Yeager (1941) has shown that in the holometabolous southern armyworm *Prodenia eridania* the glycogen content of the haemocytes falls very considerably at the end of the pre-pupal stage, and this suggests the possibility that the increase in the trehalose content of the haemolymph of *S. gregaria* during the last 24 hr of the fifth instar is due to the breakdown of the glycogen of the haemolymph.

Since the locusts which gave the 'summer' and 'winter' batch results were taken from the same stock and reared under exactly the same conditions, except for diet, it is concluded from a comparison of the various reducing values of these two groups, that variation in diet causes immediate and considerable changes in the concentration of the reducing materials in the haemolymph, but that the intrinsic patterns of variation with age are not destroyed.

The work of Heal (1948) and Beard (1949) showed that insects can quickly recover from very considerable changes in the composition of their haemolymph

and TREHERNE (1958b) has shown that absorption of glucose from the gut lumen is not only rapid but is probably facilitated by its rapid conversion to trehalose. In view of these and the above findings it is therefore suggested that quantitative measurements on haemolymph should not be made without reference to age, sex, and physiological condition of the experimental insect.

Acknowledgements—The authors wish to thank the Agricultural Research Council for the financial support which enabled this work to be carried out and the Anti-Locust Research Centre for their supply of locusts. Thanks are also due to Professor F. C. HAPPOLD for the interest he has taken in this work and the hospitality of his department.

REFERENCES

BEARD R. L. (1949) Physiological effects of induced haemorrhage in Japanese beetle larvae. *J. N.Y. ent. Soc.* **57**, 79–91.

BERKMAN S., HENRY R. J., GOLUB O. J., and SEGALOVE M. (1954) Tungstic acid precipitation of blood proteins. *J. biol. Chem.* **206**, 937.

BÜCHER T. and KLINGENBERG M. (1958) Wege des Wasserstoffs in der lebendigen Organisation. *Angew. Chem.* **70**, 552–570.

FLORKIN M. (1937) Contributions à l'étude de plasma sanguin des insectes. *Mém. Acad. R. Méd. Belg.* **16**, 1–96.

HAWKE P. B., OSER B. L., and SUMMERSON W. H. (1947) *Practical Physiological Chemistry*, p. 528. Churchill, London.

HEAL R. E. (1948) A technique for the bloodstream injection of insects. *J. econ. Ent.* **41**, 535–542.

HOWDEN G. F. and HUNTER-JONES P. (1958) An artificial diet for the laboratory rearing of locusts. *Nature, Lond.* **182**, 1527–1528.

HOWDEN G. F. and KILBY B. A. (1956) Trehalose and Trehalase in the locust. *Chem. & Ind.* 1453–1454.

HUNTER-JONES P. (1956) *Instructions for Rearing and Breeding Locusts in the Laboratory*. Anti-Locust Research Centre, London.

KUWANA Z. (1937) Reducing power of the body fluid of the silkworm. *Jap. J. Zool.* **7**, 273–303.

LEE R. (1958) Unpublished results reproduced with the author's kind permission.

TREHERNE J. E. (1958a) The absorption and metabolism of some sugars in the locust *Schistocerca gregaria* (Forsk.). *J. exp. Biol.* **35**, 611–625.

TREHERNE J. E. (1958b) The absorption of glucose from the alimentary canal of the locust *Schistocerca gregaria* (Forsk.). *J. exp. Biol.* **35**, 297–306.

YEAGER J. F. (1941) Histochemical detection of glycogen in blood cells of the southern armyworm (*Prodenia eridania*) and in other tissues, especially midgut epithelium. *J. agric. Res.* **63**, 257.

VOL. 4 1960

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

S. D. BECK and W. HANEC: Diapause in the European corn borer, *Pyrausta nubilalis* (Hübn.)

H. LANGER: Die Wirkung von Licht auf den chemischen Grundaufbau des Auges von *Calliphora erythrocephala* Meig.

R. W. NEWBURGH, L. N. POTTER, and V. H. CHELDELIN: Oxidative phosphorylation in *Phormia regina* larvae

P. W. MILES: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* Dall. (Heteroptera, Lygaeidae)—III. Origins in the salivary glands

R. H. DADD: The nutritional requirements of locusts—I. Development of synthetic diets and lipid requirements

K. U. CLARKE: Studies on the relationships between air temperature and the internal body temperature of *Locusta migratoria*

H. CHINO: Enzymatic pathways in the formation of sorbitol and glycerol in the diapausing egg of the silkworm, *Bombyx mori*—I. On the polyol dehydrogenases

L. Y. FINLAYSON: A comparative study of the effects of denervation on the abdominal muscles of saturniid moths during pupation

R. H. HACKMAN and M. GOLDBERG: Composition of the oothecae of three Orthoptera

D. S. ANDERSON: The respiratory system of the egg-shell of *Calliphora erythrocephala*

W. H. TELFER and C. M. WILLIAMS: The effects of diapause, development, and injury on the incorporation of radioactive glycine into the blood proteins of the Cecropia silkworm

M. V. BRIAN and C. A. H. CARR: The influence of the queen on brood rearing in ants of the genus *Myrmica*

K. U. CLARKE and R. W. BALDWIN: The effect of insect hormones and of 2 : 4-dinitrophenol on the mitochondrion of *Locusta migratoria* L.

G. BAKER, J. H. PEPPER, L. H. JOHNSON, and E. HASTINGS: Estimation of the composition of the cuticular wax of the mormon cricket, *Anabrus simplex* Hald.

T. ITO: Effect of sugars on feeding of larvae of the silkworm, *Bombyx mori* L.

L. BARTON BROWNE: The rôle of olfaction in the stimulation of oviposition in the blowfly, *Phormia regina*

W. HANEC and S. D. BECK: Cold hardiness in the European corn borer, *Pyrausta nubilalis*

M. LÜSCHER und F. ENGELMANN: Histologische und experimentelle Untersuchungen über die Auslösung der Metamorphose bei *Leucophaea maderae*

H. E. HINTON: Cryptobiosis in the larva of *Polypedilum vanderplanki* Hint. (Chironomidae)

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 271 *to* 282. *Pergamon Press Ltd., London. Printed in Great Britain*

THE SALIVARY SECRETIONS OF A PLANT-SUCKING BUG, *ONCOPELTUS FASCIATUS* (DALL.) (HETEROPTERA : LYGAEIDAE)—III ORIGINS IN THE SALIVARY GLANDS

P. W. MILES*

Department of Zoology, University of Cambridge

(*Received* 15 *September* 1959)

Abstract—The physical and chemical properties of the contents of the salivary glands are discussed in relation to the properties of the emitted salivary secretions, and the following conclusions are drawn. Sheath material originates as two distinct secretions, produced respectively in the anterior and lateral lobes. The precursors are elaborated as concentrated solutions of critical composition, and withdrawal or addition of water causes coagulation with hydrogen bonding; but the action of the oxidase secreted by the accessory gland is probably necessary to confer the final chemical and physical stability shown by sheath material. The watery saliva which is ejected independently of sheath material originates in the posterior lobe, but it may also contain mucoids secreted by the accessory gland or by the ducts generally.

VOL. 4 1960

INTRODUCTION

In the recent, detailed account of the anatomy and histology of the salivary glands of *Oncopeltus* by Bronskill *et al.* (1958), the terms used for the lateral and posterior lobes of the principal gland are the reverse of those established for Hemiptera generally by Baptist (1941) and Nuorteva (1956). The convention of the latter authors will be used throughout this paper (see Fig. 1), even where reference is made to the results of Bronskill *et al.*; thus where Bronskill *et al.* have made a statement about the 'lateral lobe', it will be reported here as referring to the posterior lobe, and vice versa.

Most accounts of the salivary glands of Hemiptera suggest that the contents of all the lobes of the principal gland are proteinaceous (Baptist, 1941; Nuorteva, 1954; Bronskill *et al.*, 1958). Fauré-Fremiet (1910) stated that the glands of aquatic Heteroptera produce an oily secretion, but Baptist was unable to confirm this. Baptist also disagreed with Fauré-Fremiet on the extent to which the contents of the different lobes could be differentiated by their chemical nature; however, in the salivary glands of many Heteroptera, some differences in the reactions of the contents to fixatives and stains have been noted (Baptist, 1941; Goodchild, 1952; Bronskill *et al.*, 1958).

Baptist concluded that the salivary glands of Heteroptera secrete at the most two enzymes. Both Baptist and Nuorteva (1954) claim that, in the insects they investigated, all the lobes of the principal gland produced the same types of

* Now at the Waite Agricultural Research Institute, University of Adelaide.

enzyme, and BAPTIST found no enzymes in the accessory apparatus. However, BRONSKILL *et al.* claim positive results for amylase, invertase, proteinase, and lipase in the salivary glands of *Oncopeltus*, variously distributed between the lobes of the principal gland and the accessory gland.

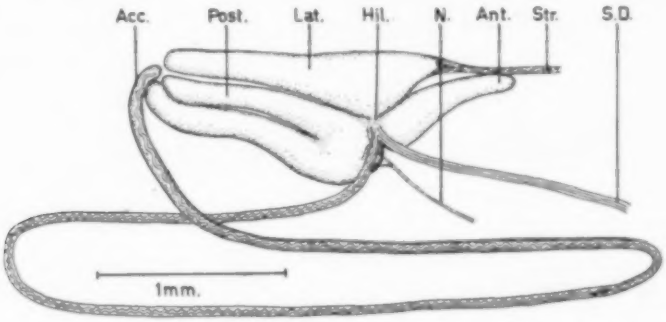


FIG. 1. Salivary gland of *Oncopeltus*. Acc., accessory gland; Ant., anterior lobe; Hil., hilus; Lat., lateral lobe; N., nerve; Post., posterior lobe; S.D., salivary duct; Str., strand of richly nucleated tissue running into the head capsule.

The present paper relates the physical and chemical properties of the contents of the different lobes of the salivary glands of *Oncopeltus* to the origin and formation of the salivary secretions ejected from the stylet bundle, viz. the sheath material and the watery saliva (MILES, 1958, 1959).

PRELIMINARY OBSERVATIONS ON THE CONTENTS OF THE UNFIXED GLANDS

The principal salivary glands of *Oncopeltus* are colourless and transparent. In distilled water, the glands gradually swelled, becoming twice their normal size within 5 min. The cells of the posterior lobe became especially distended, projecting three or four times their natural thickness into the lumen of the gland. The water which diffused into the posterior lobe mixed with the contents, although after a few minutes some opaque patches were differentiated. On the other hand, the water diffusing into the lateral and anterior lobes did not mix with the bulk of the contents, which were forced towards the hilus of the gland and became opaque where in contact with the surrounding liquid. The immiscible material in the anterior lobe eventually formed a large, discrete globule. After some time, the pressure of absorbed water forced the immiscible contents of the lateral lobe through the hilus into both the other lobes. The material from the lateral lobe then mixed with the globule in the anterior lobe, but not with the contents of the posterior lobe.

In most instances, the posterior lobe of a water-soaked gland eventually burst, shedding its own contents and the contents of the lateral lobe. The material pouring out of the gland contained numerous small, transparent droplets and soon coagulated into a rubbery but friable mass. The small droplets superficially

resembled oily droplets, but they did not rise to the surface of water and did not appear to stain with Sudan III. The non-Sudanophil nature of the droplets was confirmed in the following manner: the glands were first immersed in 50% glycerol, when the contents of the anterior and lateral lobes appeared to contract and coagulate, and the contents of the posterior lobe were then pressed out under a coverslip into an alcoholic solution of Sudan III. The main mass of the emitted material coagulated on contact with the alcohol and stained orange-brown, whereas the droplets themselves remained distinct and did not stain. It was therefore apparent that the droplets did not contain lipids.

When a fresh gland was placed in distilled water and the various lobes punctured immediately, it appeared that the contents of only the punctured lobe flowed out. The bulk of the contents of the posterior lobe at first mixed with water on leaving the gland, and within a distance of about 1 mm from the puncture was no longer distinguishable. At the edge of this region, a fine, granular precipitate was thrown down. Small amounts of other materials also emerged from the posterior lobe. Irregular lumps of matter immediately coagulated from the contents and presumably corresponded to the areas of opacity noted in the water-soaked lobe; and the 'pseudo-oily droplets', which were already present in the lobe, gradually flattened into corrugated discs. The contents of the lateral lobe, on the other hand, left the gland as a serpentine stream which did not disperse and formed an opaque skin at its surface. The material within the skin remained liquid for a while, but gradually swelled as water was absorbed, and eventually became a reticulated, sticky mass. No other material was observed to flow from the lateral lobe. The contents of the anterior lobe coagulated into irregular masses if shed immediately; but if the gland was left in water just long enough for a discrete globule of material to separate in the anterior lobe, as described above, and the lobe was punctured before any admixture with the contents of the lateral lobe had occurred, the globular mass immediately formed a thickly opaque gel which did not swell, whereas the rest of the contents showed the same characteristics as the contents of the posterior lobe. The latter fraction could conceivably have flowed in from the posterior lobe through the hilus during the short period of soaking, but the same results were obtained while the opening to the hilus appeared to remain occluded by the immiscible material, and it could well be that both fractions occur naturally in the anterior lobe.

When the gland was ruptured in a series of Ringer's solutions, buffered from pH 2 to 8, the contents of the posterior lobe were completely soluble with the exception of the pseudo-oily droplets, whereas the contents of the lateral and anterior lobes appeared to behave as they did when the gland was punctured immediately in distilled water.

The contracted and coagulated masses which formed in the lateral and anterior lobes of glands immersed in 50% glycerol showed birefringence when compressed. When the gland was treated with conc. HCl, the coagulated mass in the anterior lobe dissolved rapidly, whereas that in the lateral lobe swelled to a limited extent but remained insoluble.

VOL. 4 1960

When the glands were ruptured under white oil, the contents of all the lobes were freely miscible in one another and did not coagulate, showing that although contact with water or withdrawal of water (by placing the gland in a hypertonic solution) caused coagulation of some components, the mixing of the secretions or their mere removal from the gland did not.

From these observations, the secretions of the principal gland can be characterized as follows:

I. *Posterior lobe*

(a) a component which mixes with small amounts of water and throws down a precipitate on further dilution, but which is completely soluble in saline;

(b) a component which coagulates out into irregular masses in water but is soluble in saline;

(c) droplets which are present in the original contents and coagulate in both water and saline.

II. *Lateral lobe*

(a) a secretion which does not disperse in either water or saline but swells and coagulates, and which when coagulated in hypertonic solutions is resistant to solution in HCl.

III. *Anterior lobe*

(a, b, c) a fraction which exhibits the same behaviour in water and saline as the contents of the posterior lobe (but which may possibly have originated in the posterior lobe and have been transferred to the anterior lobe during the investigation);

(d) a fraction which coagulates but does not swell in either water or saline and is soluble in HCl.

HISTOCHEMICAL AND STAIN REACTIONS OF THE CONTENTS OF THE GLANDS

When fixed in neutral formol, the lobes of the principal gland contained colourless, rubbery masses which were occasionally birefringent. In the anterior lobe, either the contents were precipitated as a retinaculum, or part of the secretion formed a discrete mass within the lobe; and both phenomena are consistent with the presence, in subequal amounts, of two fractions such as those described above.

Histochemical tests were applied to the whole, fixed glands, and the same tests were applied to the emitted salivary secretions, as described previously (MILES, 1960). The G-Nadi procedure (PEARSE, 1953), was applied to unfixed glands and sheath material. Table 1 summarizes the properties of the contents of the three lobes of the principal gland, the accessory gland, and the two emitted secretions.

TABLE 1—REACTIONS[1] OF THE SALIVARY GLAND CONTENTS AND SALIVARY SECRETIONS OF *Oncopeltus*

Test or treatment	Anterior lobe	Lateral lobe	Posterior lobe	Accessory gland	Sheath material	Watery saliva
* Isotonic saline	Coagulates	Swells, coagulates	Soluble		Insoluble	Soluble
Conc. HCl	Dissolves	Resistant	Dissolves		Resistant	Dissolves
Millon's reagent	+	++	+		++	+
Tetrazonium	+	++	±		+	±
Benzoquinone	++	+	++		++	0
Schiff's reagent	+	+ fades	0		+ fades	0
PAS	++	0	+		+	+
PAS after acetylation	±	0	0		+	+ bluish
Toluidine blue	Pale blue-green	0	Dark blue	Violet, duct pink	Pale blue-green	Dark blue to pink
Fat stains[2]	0	+	0		±	0
Argentaffin	0	±	++		+	0
* G-Nadi	±[3] on standing	±[3] on standing	0	++[4]	+[3]	
Aniline blue in lactophenol	+	++	0		+	0
Isoelectric point	5·4	5·4	5·4		5·4	

The reactions of the cell contents of the accessory gland have been noted only where they differed from those of the walls of the principal gland. Tests marked * were made on fresh material, the others on formol-fixed material. [1] ++, strongly positive; +, positive; ±, weakly positive or variable; 0, negative. [2] Sudan black B, Sudan III, Sudan IV. [3] Not inhibited by cyanide. [4] Inhibited by cyanide but not by azide.

VOL. 4 1960

The posterior lobe

The contents of the posterior lobe gave a positive PAS reaction which was destroyed by prior acetylation and restored by alkaline hydrolysis; it is therefore probable that carbohydrate is present. The contents gave a positive reaction for tyrosine and were strongly coloured by benzoquinone, suggesting the presence of protein.

The walls of the salivary glands rapidly took up toluidine blue but, on standing, the contents of only the posterior lobe appeared to stain orthochromatically. This reaction was quite different from the characteristic pale greenish-blue produced in sheath material, whereas watery saliva takes up the stain strongly, giving a range of colours from dark blue to violet and pink (MILES, 1959).

The contents of the posterior lobe gave a strongly positive argentaffin reaction which was not affected by prior incubation in bisulphite, or by fixation for 6 days in neutral formol. Tests for diphenols were negative. The argentaffin reaction of sheath material, either fresh or after treatment with formol, was significantly weaker; and, in formol-fixed agar gels containing both sheath material and watery saliva, no material could be found which was as strongly argentaffin as the contents of the posterior lobe. On the other hand, such a material was often found trapped in the central canal of unfixed mounds of sheath material which had been secreted onto glass. The trapped material may have been watery saliva; and, since the agar gel preparations contained watery saliva in thin, diffuse layers which would

have been readily penetrated by the formol during fixation, it is possible that only that part of the secretion which had its argentaffin groups (e.g. polyamines or aminophenols) thoroughly blocked by formol remained after the fixative had been washed out. The failure of fixed watery saliva to give a reaction with benzoquinone, which strongly coloured the contents of all the lobes of the salivary gland, lends support to this view.

The lateral lobe

The contents were PAS-negative, indicating the absence of carbohydrate. Tests for tyrosine and the reaction with benzoquinone were positive, suggesting the presence of protein. When the gland was treated with Sudan III for 15 min, rinsed in 50% alcohol, and mounted in glycerine jelly, the lateral lobe stained red and the other lobes orange-brown. After a month, only the lateral lobe had retained its colour, which has since persisted for over 3 yr. No confirmatory tests for lipid were carried out, but in view of the indications of lipid in sheath material, the ability of the contents of the lateral lobe to stain with Sudan III may be significant.

The contents were colourless immediately after application of the full Nadi procedure. However, if the gland was left in the final saline rinse, the contents of the lateral and anterior lobes, but not of the posterior lobe, developed a weak colour overnight, even in the presence of cyanide; whereas no colour developed if the gland was confined with some of the final rinse under a coverslip. This suggests that the contents of the lobe are able, to a limited extent, to take up free oxygen non-enzymatically; it may also explain the reaction with Schiff's reagent in the formol-fixed gland, since this was not affected by incubation with bisulphite to block aldehydes and was probably due to reoxidation of the leucofuchsin. Sheath material is Nadi-positive when emitted and also stains with Schiff's reagent.

The readiness with which the material in the lateral lobe coagulates on the withdrawal or addition of water strongly suggests that it enters into the composition of sheath material. This is corroborated by the ability of the material to stain with Sudan III, its resistance to solution in HCl, its moderate argentaffin reaction, and its reactions with Nadi reagent and Schiff's reagent.

The anterior lobe

The contents stained with Schiff's reagent, before and after incubation with bisulphite, and gave a strong PAS reaction which was decreased by acetylation and restored by alkaline hydrolysis. This suggests that some carbohydrate is present in addition to a substance which can reoxidize leucofuchsin in the formol-fixed gland. The material also stained weakly with Nadi reagent after standing overnight in saline, with or without cyanide. Confirmatory tests for protein were positive. The secretion gave no argentaffin reaction, but stained the same pale greenish-blue with toluidine blue as sheath material.

From these reactions, it seems likely that at least part of the contents of the anterior lobe enters into the sheath material.

The accessory gland

The cells of the glandular part of the accessory apparatus showed the same general properties as the walls of the principal gland with two significant exceptions.

When the whole gland was placed in 0·01% toluidine blue, the accessory gland alone showed beta metachromasia, while the intima of the ducts of both accessory and principal glands showed gamma metachromasia. This suggests that the accessory gland, and possibly the ducts in general, secrete mucoid substances.

The accessory gland gave a strong Nadi reaction except in the presence of cyanide; azide did not inhibit the reaction, and this could indicate the presence of a polyphenol oxidase (BRUNET, 1952). In view of the strong Nadi reaction of sheath material when it is first secreted, and the indication that the contents of the lateral and anterior lobes may take up some oxygen in the absence of enzymes, it is possible that the oxidase of the accessory gland acts on these secretions as they are emitted. However, if mucoid substances are also secreted by the accessory gland, these presumably find their way into the watery saliva.

VOL. 4 1960

STAIN REACTIONS OF THE CONTENTS REMOVED FROM UNFIXED GLANDS

Tests on fixed, whole glands did not differentiate components produced in the same lobe, and an attempt was therefore made to remove the contents of individual lobes of fresh glands and apply to them stains which differentiated regions in the stylet sheath or fixed watery saliva (MILES, 1959, 1960). The separation of lobes has been reported previously (e.g. by BAPTIST and BRONSKILL *et al.*) without special reference to technique, but it was found here that when a fresh gland was divided anywhere, the contents tended immediately to bathe the entire gland. Moreover, it is possible that pressure on one lobe could cause its contents to flow into the other lobes through the hilus. Eventually the following method was adopted: a fresh gland was held by the salivary duct, rinsed rapidly in Ringer's solution, and the liquid drained away with filter paper; one lobe was held lightly with forceps while its base was blocked off at the hilus with a microscalpel; the lobe was then quickly pulled away and laid on a slide. The contents were pressed out and the walls of the empty lobe, together with the rest of the gland, were discarded. The expressed contents were allowed to dry, and were then fixed in methyl alcohol for 2 min. Finally, the alcohol was allowed to evaporate completely.

The contents of all the lobes invariably separated on drying into a mass of relatively thick material surrounded by a thinner film of secretion. The lateral lobe contained a relatively large amount of 'bulky secretion' and only a small quantity of 'thin secretion' while, conversely, a thin secretion was predominant in the posterior lobe. The two types of secretion were present in subequal amounts in the contents of the anterior lobe.

When stained with safranin followed by light green, the bulky masses took up the former, and the surrounding films the latter; although the bulky secretion

of the anterior lobe stained purple, indicating that some light green had also been taken up.

In toluidine blue, the bulky mass from the lateral lobe did not stain, and that from the anterior lobe became a pale greenish-blue. The small amounts of bulky secretion from the posterior lobe were overlaid and masked by the relatively large quantity of thin secretion. The thin secretions from all lobes stained strongly with toluidine blue; weakly metachromatic patches occurred, chiefly in the secretion from the anterior lobe. None of the materials showed the degree of metachromasia found in the watery saliva and in the accessory gland. Immersion of fixed whole glands in toluidine blue did not appear to demonstrate orthochromatic materials except in the posterior lobe; but it is possible that, during formol-fixation, the 'thin' fractions in the anterior and lateral lobes tended to separate out peripherally and were subsequently masked by the strongly staining walls of the gland.

SALIVARY ENZYMES

Fresh salivary glands were rinsed in Ringer's solution and ground with 0·05 ml distilled water per pair of glands. Half the extract was immersed for 10 min in boiling water and used in all tests as a control.

For proteinase and amylase, the methods of Pickford and Dorris (1934) were used. To test for other carbohydrases, 0·05 ml extract was incubated with 0·05 ml 1 per cent substrate and 0·05 ml phosphate buffer, pH 5·0, for 2 hr at 37°C; activity was revealed with acid copper reagent (Tauber and Kleiner, 1932) and Nelson's reagent (Nelson, 1944). For lipase, the method used was essentially that of Cole (1933): 0·1 ml olive oil emulsion containing phenol red was added to 0·1 ml extract, the pH was adjusted to 7·8 with sodium carbonate, and the mixtures were incubated at 37°C.

No proteinase activity was found at pH 4·8, the optimum pH for the proteinase found in the gut (Miles, unpublished data), although Bronskill *et al.* recorded strong proteolytic activity in the lateral lobe at pH 4·2 and 5·3.

Amylase was present, and by transferring the contents of individual lobes directly to the starch-agar slide, it was found to be localized in the posterior lobe. Bronskill *et al.* also found a strong reaction in the posterior lobe, and weaker activity in the lateral lobe.

No lipase was detected in the salivary glands although, by comparison, one region of the gut gave a positive reaction within 24 hr. However, the technique used was relatively insensitive compared with the histochemical method used by Bronskill *et al.* who found a lipase in the lumen of the lateral lobe. It is noteworthy that this is the lobe which gives an indication of the presence of lipid.

The gland as a whole gave positive reactions for invertase and maltase, but not for β-glucosidase (using cellobiose as a substrate), α-galactosidase (using melibiose), β-galactosidase (using lactose), pectin esterase (using citrus pectin), polygalacturonase (using low-methoxy pectin), nor for cellulase (using carboxy-methyl-cellulose). Localization of the invertase and maltase was not attempted. Bronskill *et al.* tested for invertase and found it in the posterior lobe, and to a lesser extent in the anterior and lateral lobes.

DISCUSSION

There seems little doubt that sheath material is derived mainly from the bulky, coagulating components produced in the lateral and anterior lobes; and both appear to participate since the properties of the sheath are shown by one or other of the secretions but cannot be derived from either alone. The possible relation of the oxidase secreted in the accessory gland to sheath material will be discussed below.

The secretion of the posterior lobe must form at least part of the watery saliva since the latter contains an amylase (MILES, 1959), and the posterior lobe is the source of most, if not all, of this enzyme in the gland. However, the watery saliva often contains substances (presumably mucoids) which show strong beta or gamma metachromasia, and these must originate elsewhere. The accessory gland is the only source of material showing beta metachromasia of similar intensity, and the only parts of the salivary apparatus showing gamma metachromasia are the ducts of both the principal and the accessory glands. BAPTIST concluded that the accessory gland in Hemiptera is 'purely a development of the primary conducting part of the glandular system', whereas DAY (1951) suggested that the conducting part of the salivary system of the cockroach changes the nature of the saliva as it passes; and it is possible that, in *Oncopeltus*, not only the glandular part of the accessory gland but also the ducts in general secrete mucoids.

VOL. 4 1960

While it is relatively easy to locate the origin of some components of the emitted saliva, it is difficult to assign all the components produced within the gland to one or other of the emitted secretions. The bulky coagulating secretions of the lateral and anterior lobes are unlikely to participate in the watery saliva, since they coagulate immediately on dilution with saline and watery saliva is emitted as a clear, homogeneous liquid which is freely miscible with saline. However, the status of the water-dispersible fraction from the anterior lobe is obscure. It has been suggested above that this fraction may in fact flow in from the posterior lobe during investigation of the gland; however, the presence of a 'thin secretion' in the contents removed from the fresh anterior lobe, and the indication of a carbohydrate in the formol-fixed lobe, tend to confirm the presence of the fraction in the original contents. It is difficult to see how the two fractions in the anterior lobe could be discharged independently, since they are originally present as an intimate mixture. It is probable that sheath material is always ejected in company with the water-dispersible fraction from the anterior lobe, together with the 'thin secretion' which is found in small quantities in the lateral lobe; these may function as lubricants or serve to maintain the sheath-precursors in solution until after ejection, and then diffuse away. The watery saliva which is very often secreted independently of sheath material (MILES, 1959) must have an independent source, and evidently originates in the posterior lobe. The small quantity of material in this lobe which coagulates in contact with water is presumably that which stains with safranin when the contents are removed from the fresh lobe, and it may form the relatively insoluble component of watery saliva which is fixed as

a retinaculum and stains similarly in agar gel. However, the status of the 'pseudo-oily droplets' remains problematical.

BRONSKILL *et al.* state that the glands have two independent sources of innervation, one supplying the posterior lobe and accessory apparatus and the other supplying the anterior and lateral lobes. There is thus no difficulty in accounting for the independent discharge of the secretions of the posterior lobe and accessory gland to produce watery saliva and of the anterior and lateral lobes to produce sheath material. However, from studies on the ejection of salivary materials by *Oncopeltus* (MILES, 1959), it would seem likely that, although watery saliva can be ejected independently, sheath material is normally ejected in company with watery saliva; and this would account for the possible participation of a secretion from the accessory gland in sheath formation.

If the contents of the lateral lobe are concerned in the production of sheath material, it is difficult to account for the enzymes found in this lobe by BRONSKILL *et al.* Unless very great care was exercised in the separation of the lobes, it is possible that the amylase and invertase found by BRONSKILL *et al.* in the lateral lobe could have been contaminants originating in the posterior lobe; but proteinase and lipase were found by them in the lateral lobe alone. The latter enzymes may conceivably be concerned in the elaboration of sheath precursors within the gland, since it is possible that lipid as well as protein is contributed to the sheath by the secretions of the lateral lobe; alternatively, if some watery saliva is secreted with sheath material, these enzymes may become incorporated in the former when the sheath material separates out. Nevertheless, if proteinase and lipase are secreted solely in the lateral lobe, it must be assumed that they are absent from the relatively large quantity of watery saliva which may be discharged independently during feeding.

The PAS reactions of the contents of the principal gland indicate that carbohydrate is absent in the lateral lobe but present in the posterior and possibly also in the anterior lobe. This carbohydrate may be ascribable to mucoid substances, in view of the weakly metachromatic materials which occur in the principal gland, but it could also be associated with enzymes (VEIBEL, 1951). After the acetylation procedure, the anterior lobe shows an anomalous reaction which partially masks a true carbohydrate reaction; and the anomalous reaction is similar to that shown by sheath material (MILES, 1960). The reaction of fixed watery saliva after acetylation, although also anomalous, is quite distinct; however, the secretion was examined in agar gel, and the intimate mixture of the secretion with agar may possibly have masked the true reaction of the former.

The stabilization of sheath material has been discussed previously (MILES, 1960). It is evident that the anterior and lateral lobes contain the precursors in solutions of critical composition, since the contents of these lobes begin to coagulate where in contact with small amounts of water diffusing into the gland from hypotonic solutions, and they coagulate completely when the gland is immersed in a hypertonic solution. This may be due entirely to the addition or extraction of water; it is known that some proteins remain in solution only at

critical concentrations at which the protein itself maintains a high enough dielectric constant to prevent hydrogen bond formation, whereas at lower concentrations the dielectric constant is too low, and at higher concentrations the proximity of the molecules promotes bond formation. It is also possible that the sheath precursors are kept in solution in the gland by salts, or by other dipole molecules such as peptides or amino acids ('salting in'). However, the secretions did not remain in solution when diluted with isotonic saline or glycine; and it is evident that once discharged by the insect, the sheath precursors will coagulate, due either to dilution or to loss of water or other accompanying diffusable substances, depending on the substrate.

Thus it does not seem necessary to postulate that, in the natural process of coagulation of sheath material, a muscular press causes unfolding and alignment of the molecules as in the formation of silk (COLEMAN and HOWITT, 1947). Pressure is presumably exerted in the salivary pump, and molecular alignment may conceivably occur as the secretion is forced down the narrow salivary canal of the stylets; yet sheath material is not extruded as a thread, nor does it necessarily show much birefringence, although more can be induced by pressure on the already hardened mass. Nevertheless, mere mixing of the secretions of the principal gland, whether under water or in air, does not produce a substance comparable with sheath material; and in view of the evidence for chemical bonding in addition to H-bonding in the latter (MILES, 1960), it is tempting to suggest that the oxidase of the accessory gland produces some form of oxidative bonding, possibly 'self-tanning', in the final product.

VOL. 4 1960

Acknowledgements—This paper represents part of a thesis submitted for the degree of Ph.D. in the University of Cambridge; and my sincere thanks are due to my supervisor, Professor V. B. Wigglesworth, and to Professor Sir James Gray for providing laboratory facilities. I am also grateful to Rhodes University, South Africa; to the trustees of the Southern Rhodesian State Lotteries; and to the trustees of the Charelick Salomon Scholarship Fund, Southern Rhodesia for scholarships and grants which made the work possible.

REFERENCES

BAPTIST B. A. (1941) The morphology and physiology of the salivary glands of Hemiptera-Heteroptera. *Quart. J. micr. Sci.* **82**, 91–193.

BRONSKILL J. F., SALKELD E. H., and FRIEND W. G. (1958) Anatomy, histology, and secretions of salivary glands of the large milkweed bug, *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). *Canad. J. Zool.* **36**, 961–968.

BRUNET P. C. J. (1952) The formation of the ootheca by *Periplaneta americana*—II. The structure and function of the left colleterial gland. *Quart. J. micr. Sci.* **93**, 47–69.

COLE S. W. (1933) *Practical Physiological Chemistry*. Heffer, Cambridge.

COLEMAN D. and HOWITT F. O. (1947) Studies on silk proteins—I. The properties and constitution of fibroin. The conversion of fibroin into a water-soluble form and its bearing on the phenomenon of denaturation. *Proc. roy. Soc.* (A) **190**, 145–169.

DAY M. F. (1951) The mechanism of secretion by the salivary gland of the cockroach *Periplaneta americana* L. *Aust. J. sci. Res.* (B) **4**, 136–143.

FAURÉ-FREMIET E. (1910) Glandes labiales des Hydrocorises. *Ann. Sci. nat. Zool.* (9) **12**.

GOODCHILD A. J. P. (1952) A study of the digestive system of the West African cacao Capsid bugs. (Hemiptera, Miridae.) *Proc. zool. Soc. Lond.* **122**, 543–572.

MILES P. W. (1958) Contact chemoreception in some Heteroptera, including chemoreception internal to the stylet food canal. *J. Ins. Physiol.* **2**, 338–347.

MILES P. W. (1959) The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera: Lygaeidae)—I. The types of secretion and their roles during feeding. *J. Ins. Physiol.* **3**, 243–255.

MILES P. W. (1960) The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (Heteroptera: Lygaeidae)—II. Physical and chemical properties. *J. Ins. Physiol.* **4**, 209–219.

NELSON N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375–380.

NUORTEVA P. (1954) Studies on the salivary enzymes of some bugs injuring wheat kernels. *Ann. ent. fenn.* **20**, 102–124.

NUORTEVA P. (1956) Studies on the comparative anatomy of the salivary glands in four families of Heteroptera. *Ann. ent. fenn.* **22**, 45–54.

PEARSE A. G. E. (1953) *Histochemistry: Theoretical and Applied.* Churchill, London.

PICKFORD G. E. and DORRIS F. (1934) Micromethods for the detection of proteases and amylases. *Science* **80**, 317–319.

TAUBER T. and KLEINER I. S. (1932) A method for the determination of monosaccharides in the presence of disaccharides and its application to blood analysis. *J. biol. Chem.* **99**, 249–255.

VEIBEL S. (1951) β-Glucosidase. *The Enzymes*, pp. 583–620. (Edited by SUMNER J. B. and MYRBÄCK K.) Academic Press, New York.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 283 *to* 303. *Pergamon Press Ltd., London. Printed in Great Britain*

DIE WIRKUNG VON LICHT AUF DEN CHEMISCHEN GRUNDAUFBAU DES AUGES VON *CALLIPHORA ERYTHROCEPHALA* Meig.*

HELMUT LANGER

Zoologisches Institut der Universität München

(*Received* 26 *November* 1959)

Abstract—The fundamental chemical composition of the compound eye of insects was analysed using the blow-fly, *Calliphora erythrocephala*, as an example. The variations in chemical composition caused by long-continued action of visible and ultra-violet light on the living animals were investigated. The material of isolated eyes was fractionated by several methods, the amounts of the important groups of substances and the fractions of nitrogen- and phosphorus-containing compounds, as well as of carbohydrates, were estimated. The small amount of DNA (the ratio of RNA : DNA is about 3·4) and the large amount of carbohydrates, especially glycogen, are remarkable.

Several hours' irradiation of whole animals has no influence on the quantity of all building substances (e.g. total protein, nucleic acids, lipids) investigated in the eyes. Under the same conditions, the following changes in the amounts of metabolic substances are present, all of which demonstrate an increase in production of energy: the quantity of total carbohydrates is diminished, the portion of free oligosaccharides is increased, and the content of energy-rich phosphorus compounds shows a reduction. The effects of visible and ultra-violet light are in the same direction, but UV is more efficient than visible light.

It is concluded from results that there is a higher turnover in energy-producing metabolism during adequate stimulation. This phenomenon is to be related to the intracellular processes which occur in connexion with the conversion of photic stimuli into neural excitation in the sensory cells.

VOL. 4 1960

Der Beitrag, der durch chemisch-physiologische Untersuchungen zur Aufklärung der Funktion des Facettenauges geleistet werden kann, erstreckt sich ebenso wie bei der Retina des Linsenauges auf zwei verschiedene Probleme. Einerseits besteht die Frage nach den primären Prozessen der Reiztransformation in der Sinneszelle, für die spezifische Farbstoffe eine entscheidende Rolle spielen. Andererseits ist zu klären, auf welchen energetischen Grundprozessen sich diese Vorgänge aufbauen und woher die für die Bildung der Erregung der Sinneszelle nötige Energie kommt.

Am Facettenauge sind in den letzten Jahren Ansätze zur Erforschung des ersten Problems durch Untersuchung der Sehfarbstoffe gemacht worden (Kampa, 1955; Wald und Hubbard, 1957; Goldsmith, 1958a, b). Die dabei erfolgten Nachweise von Rhodopsin-ähnlichen Verbindungen bei Krebsen und bei der

* Die Durchführung dieser Untersuchungen wurde ermöglicht durch Mittel, die Herrn Prof. Dr. Autrum von der Deutschen Forschungsgemeinschaft zur Verfügung gestellt wurden.

Biene geben Anlass zu der Vermutung, dass sich die primären Vorgänge in den Sinneszellen des Facettenauges nicht grundsätzlich von denen unterscheiden, die für die Retina des Linsenauges angenommen werden; jedoch stehen hierüber experimentelle Untersuchungen noch aus. Über den chemischen Aufbau des Organs wurde—am Beispiel des Auges von *Calliphora*—im einzelnen in einer vorangegangenen Arbeit (LANGER 1960) berichtet, ebenso über die Veränderungen, denen die Mengen verschiedener Substanzen in Abhängigkeit vom Imaginalalter unterliegen. Über die Stoffwechselleistung des Insekten-Auges in Zusammenhang mit seiner Funktion ist bisher nichts bekannt. Mit dieser Frage soll sich die vorliegende Arbeit beschäftigen.

Für die Wirbeltier-Retina sind im Hinblick auf beide genannten Fragestellungen bereits umfangreiche Untersuchungen durchgeführt worden. Auf Grund der Versuche an den photolabilen Farbstoffen lassen sich über die Primärvorgänge beim unbunten Sehen in den Stäbchen gut fundierte Vorstellungen bilden, in denen dem Rhodopsin eine zentrale Rolle zuerkannt wird (Zusammenfassungen WALD, 1953; COLLINS, 1954; BRINDLEY, 1958), während die Kenntnisse über die Prozesse beim Farbensehen noch gering sind. Für den Grundstoffwechsel wurde in einer Anzahl von Arbeiten übereinstimmend gezeigt, dass hoher Sauerstoffverbrauch und grosse Glykolysefähigkeit vorliegen (Zusammenfassungen SÜLLMANN, 1956; PIRIE und VAN HEYNINGEN, 1956). Die Ergebnisse von Experimenten, in denen die Wirkung von Licht auf den Stoffwechsel untersucht wurde, weisen jedoch zum Teil erhebliche Diskrepanzen auf (Literatur siehe Seite 297). Diese dürften zumindest teilweise darauf zurückzuführen sein, dass der Anteil einzelner histologischer Elemente—z. B. die Summe der Stäbchen—im Verhältnis zur ganzen Retina so gering ist, dass kleinere lichtabhängige Veränderungen, die nur in einem von ihnen auftreten, am Gesamtmaterial nicht in messbarer Weise erscheinen.

Im Hinblick auf die histologische Struktur des zu analysierenden Materials bietet sich bei den Augen der Arthropoden eine günstigere Situation. Sie enthalten nur wenige Zelltypen und haben einen homogenen Aufbau; die lichtempfindlichen Zellen machen einen verhältnismässig grossen Anteil am Gesamtmaterial aus. Von vielen Insekten-Augen lassen sich die Schaltneurone des ersten optischen Ganglion durch Präparation weitgehend von der Retina abtrennen, so dass dann ausser den Sehzellen im wesentlichen nur die Stütz- und Pigmentzellen und das Material der Corneae und der Kristallkegel verbleiben, das wahrscheinlich nur wenig stoffwechselaktiv ist. Ausser diesen mikroanatomischen Gegebenheiten kommt als weiterer Vorteil hinzu, dass die Sehelemente im Appositionsauge Reaktionen auf einen breiten Intensitätsbereich der einfallenden Strahlung zeigen, da eine Adaptation durch Verminderung der Reizmenge mittels des dioptrischen Apparates—wie z. B. durch die Iris bei vielen Linsenaugen—nur in sehr geringem Umfang möglich ist. So gelangt auch bei maximaler Intensität des experimentell verwendeten Reizlichtes eine maximale Intensität zu den Sehzellen. Eine Schädigung durch "Blendung" kann dabei kaum auftreten. Denn das Facettenauge ist auch zur Raumorientierung durch Ortung des Sonnenstandes befähigt; dabei fällt die volle Helligkeit des Sonnenlichtes auf einige wenige Ommatidien. Nach einer Richtungsänderung des Tieres verarbeiten diese den dann stark veringerten Reiz ohne längere Adaptationszeit (AUTRUM, 1950).

Wenn durch Licht der Stoffwechsel der Sehzellen im Facettenauge beeinflusst wird, so ist auf Grund der vorliegenden Verhältnisse zu erwarten, dass die Veränderungen im Stoffbestand biochemisch-analytisch erfassbar sind. Aus der Kenntnis der Stoffgruppen, an denen die Veränderungen vor sich gehen, ist ein Hinweis auf die Prozesse zu erhalten, die an der Erregungsbildung der Sinneszellen direkt oder indirekt beteiligt sind.

MATERIAL UND METHODEN

Die für die Versuche verwendeten Schmeissfliegen (*Calliphora erythrocephala* Meig.) wurden in Käfigen von 50 × 30 × 30 cm Grösse in Klimakammern gehalten, die einen Tag–Nacht–Rhythmus von Temperatur, Feuchtigkeit und—beim grössten Teil der Zuchten—auch der Beleuchtung aufwiesen. Tiere eines Käfigs hatten stets ein bis auf wenige Stunden gleiches Imaginalalter. Für die Analysen wurden die Augen aus den Köpfen herauspräpariert und von den optischen Ganglien getrennt. Einzelheiten der Methoden von Zucht und Präparation siehe bei LANGER (1960).

Für die Bestrahlung mit dem sichtbaren Teil des Spektrums wurde das Licht einer 500 W—Projektionslampe mittels $CuSO_4$–Lösung und Wärmeschutz–Interferenzfilter ("Calflex"–Glas) auf den Bereich von 400–800 mμ gefiltert und auf einer Fläche von 7 cm ϕ vereinigt. Dort war ein rundes Glasgefäss gleichen Durchmessers, das mit dünner, weitmaschiger Drahtgaze verschlossen war, als Tierkäfig angebracht. Die Bestrahlungsdauer betrug 4 Std.—Die meisten Bestrahlungsversuche wurden mit einem Mischlicht mit überwiegend ultravioletten Anteilen durchgeführt. Als Lichtquelle diente ein stabförmiger Quecksilberbrenner (Typ NN 15/44 VK, 'Hanau'), dessen Emissionsbereich die Linien zwischen 254 und 436 mμ umfasst; die Strahlung wurde ungefiltert verwendet. Die mit Drahtgaze verschlossenen, länglichen Tiergefässe aus Porzellan befanden sich in 9 cm Abstand von dem mit einem Reflektor versehenen Brenner. Die Bestrahlungszeit war 6 Std. Bei allen Bestrahlungsversuchen wurden die Tiere mit Wasser und Rohrzucker versorgt.

Alle Bestimmungen bei der Analyse der Lichtwirkung wurden vom Frischmaterial ausgehend durchgeführt. Da eine genaue Ermittlung des Frischgewichts wegen zu grosser Verdunstung während der Präparation nicht möglich ist, wurden die Analysenergebnisse entweder nach direkter Bestimmung des Stickstoffs im gleichen Ansatz oder durch Umrechnung über die Anzahl der verwendeten Augen auf die Menge des säureunlöslichen Stickstoffs bezogen.

Die Fraktionierung der Phosphorverbindungen erfolgte nach SCHNEIDER (1945); die Trichloressigsäure-(TCA-) Extrakte wurden mit 5%ig wässriger Lösung bei +2° (2 Std.) und bei +90° (30 Min) hergestellt, die Lipoide durch dreimaliges Siedenlassen (je 3 Min) mit Äther–Alkohol 1 : 3 extrahiert.

Die Gesamt-Stickstoffbestimmung erfolgte durch Destillation des veraschten Materials (PARNAS und WAGNER, 1921) und colorimetrische Bestimmung des Ammoniaks mit NESSLERS Reagens in 2 aliquoten Anteilen des Destillates.—Der Gesamt-Phosphor wurde nach Veraschung colorimetrisch nach FISKE und SUBBAROW (1925) bestimmt. Für die Analyse des freien anorganischen Phosphors und die in n HCl hydrolytisch abgespaltene Orthophosphorsäure wurde die Methode von HORWITT (1952) benutzt. Die Reaktion erfolgt auch in Gegenwart von 0,5 ml n HCl je Ansatz.

Die Zuckerbestimmung geschah nach NELSON (1944). Zur Erfassung des Gesamt-Kohlenhydrats wurde mit n H_2SO_4 nach MEYERHOF (1931) hydrolysiert und mit $Cd(OH)_2$ enteiweisst (FUJITA und IWATAKE, 1931); zur Alkalisierung der Lösung wurde $BaCO_3$ verwendet. Die Fraktion des freien Zuckers wurde nach Inkubation des Gesamtmaterials mit Wasser (40°, 2 Std.), dem 1% NaF zugesetzt war, durch direkte Zuckerbestimmung im Extrakt erfasst; dabei wird auch die dann encymatisch aufgespaltene Trehalose des Gewebes mit als reduzierender Zucker erfasst (LANGER, 1959).—Die Ribosebestimmung erfolgte

nach MEJBAUM (1939); für freie Nucleotide und für RNS waren zwei Eichkurven nötig, um der verschiedenen Reaktion der Verbindungen im Orcin-Test Rechnung zu tragen (LANGER, 1956).—Die Milchsäure wurde colorimetrisch mit *p*-Hydroxy-diphenyl bestimmt (HULLIN und NOBLE, 1953), zwei Enteiweissungen reichten aus, um die Brenztraubensäure bis auf einen konstanten Rest zu entfernen. Das zur Berechnung der Ergebnisse nötige Korrekturglied wurde für diese Bedingungen bestimmt und in weitgehender Übereinstimmung mit den Angaben von HULLIN und NOBLE gefunden.

ERGEBNISSE DER EXPERIMENTE

Die Versuche wurden am Auge von *Calliphora erythrocephala* Meig. durchgeführt, dessen Funktion durch elektrophysiologische Untersuchungen (AUTRUM, 1950, 1955; AUTRUM und STUMPF, 1953; WALTHER und DODT, 1957, 1959; HOFFMANN, 1960) gut bekannt ist. Sein chemischer Grundaufbau wurde in einer vorangegangenen Arbeit näher untersucht (LANGER, 1960); die dabei erhaltenen Werte sind die Basis für die hier darzustellenden Analysen der stofflichen Veränderungen durch Lichteinwirkung.

Die wichtigsten Daten der Gesamtanalyse als Normalwerte für Tiere von mittlerem Lebensalter sind in Tab. 1 zusammengestellt. Die Analysenergebnisse wurden, da eine Bestimmung des Frischgewichts wegen der Verdunstung während der Präparation nicht möglich ist, zunächst auf je ein Auge bezogen. Dies ist angängig, weil die Tiere unter den konstanten Aufzuchtbedingungen ziemlich gleichmässig gross sind und die Augen deshalb nur eine geringe Streuung im Trockengewicht aufweisen; dieses wurde an einer grossen Zahl von Augen bestimmt. Daneben wurde der durchschnittliche Gehalt der Augen an säureunlöslichem Stickstoff in einer Anzahl von Analysen ermittelt; er ist während des gesamten Imaginallebens konstant und hat folgende Werte:

Männchen: $22{,}15 \pm 0{,}357\gamma$/Auge (46 Bestimmungen),
Weibchen: $18{,}44 \pm 0{,}302\gamma$/Auge (42 Bestimmungen).

Die erhaltenen Analysendaten wurden auf beide Bezugswerte—Gesamttrockensubstanz (GTS) und säureunlöslichen Stickstoff—umgerechnet.

Die Grössen der Augen von Männchen und Weibchen verhalten sich nach dem Gesamttrockengewicht ebenso wie nach dem Stickstoffgehalt zueinander wie 6 : 5. Das Männchen–Weibchen–Verhältnis von 1,2 tritt bei den meisten Substanzgruppen auf; für diese lässt sich ein gemeinsamer Mittelwert für beide Geschlechter berechnen. Eine Ausnahme hiervon machen einige Fraktionen der Kohlenhydrate, von denen in den Augen der Männchen wesentlich weniger enthalten ist als in denen der Weibchen.

Der säureunlösliche Stickstoff umfasst Eiweiss- und Chitin-N. Aus dem alkaliunlöslichen Anteil (20,8 γN/mg GTS) wurde der Chitingehalt ermittelt; er macht fast ein Drittel der GTS aus. Der übrige Teil (63,6 γN/mg GTS) ergibt nach Umrechnung mit dem Faktor 6,25 einen Eiweissgehalt des Augenmaterials von etwa 40% der GTS. Demnach verteilt sich der Gesamt-Stickstoff auf die Fraktionen überschlägig in folgenden Prozentsätzen: 55–60% Eiweiss-N, knapp 20% N in Chitin und etwa 20% in den leicht säurelöslichen Anteilen, etwa 5% Lipoid-N.

TABELLE 1—ÜBERSICHT ÜBER DIE ERGEBNISSE DER GESAMTANALYSE DES AUGES.–MITTELWERTE AUS JEWEILS MEHREREN BESTIMMUNGEN AN ISOLIERTEN AUGEN VON MÄNNLICHEN UND WEIBLICHEN TIEREN VON 15–21 TAGEN IMAGINALALTER UND MITTELWERTE ÜBER BEIDE GESCHLECHTER, IN γ/MG GESAMTTROCKENSUBSTANZ UND IN γ/MG SÄUREUNLÖSLICHEN STICKSTOFF BERECHNET

	♂ γ/Auge	♀ γ/Auge	Mittelwert γ/mg GTS	Mittelwert γ/mg N
Gesamttrockensubstanz	267	222		
Mengen der Fraktionen				
Leicht säurelösliches Material	23,0	18,2	84	
Gesamt-Lipoide	28,6	32,0	126	
Eiweisse	128	103	471	
Gesamt-Nucleinsäuren	4,0	3,3	15	
Chitin	87,6	69,3	320	
Glührückstand	15,4	10,1	52	
Stickstoff				
Gesamt-N	29,9	25,1	112,5	
Leicht säurelöslicher N	5,3	4,5	20,1	
Säureunlöslicher N	22,6	18,7	84,4	
Phosphor				
Gesamt-P	1,74	1,46	6,55	78,3
Gesamter leicht säurelöslicher P	0,628	0,545	2,40	28,8
davon:				
Freier anorganischer P	0,192	0,150	0,70	8,4
Freier P nach 7 Min Hydrolyse	0,382	0,337	1,48	17,6
Lipoid-P	0,825	0,690	3,08	37,2
Eiweiss-P	0,467	0,376	1,72	20,6
davon:				
Heiss-TCA-löslicher P	0,340	0,275	1,26	15,1
Heiss-TCA-unlöslicher P	0,113	0,090	0,42	5,0
Nucleotide				
Freie Nucleotide	3,4	2,8	12,7	150
Ribonucleinsäuren	3,1	2,6	11,7	140
Zucker				
Gesamt-Zucker nach Hydrolyse	9,56	12,22	♂ 35,8; ♀ 55,1	♂ 432; ♀ 653
Gesamter wasserlöslicher Zucker	8,90	10,96	♂ 33,3; ♀ 49,4	♂ 402; ♀ 586
Freier Zucker	4,61	4,40	18,6	222
Wasserunlöslicher Zucker	0,60	0,56	2,4	28,5
Reserve-Kohlenhydrate	4,35	7,26	♂ 16,2; ♀ 32,6	♂ 196; ♀ 388
Milchsäure	0,49	0,29	1,58	19,6

Aus dem Lipoidphosphorgehalt lässt sich berechnen, dass die Phosphatide nahezu die Hälfte der Gesamt-Lipoide ausmachen. Von den Gesamt-Nucleinsäuren, die aus der Menge des heiss-TCA-löslichen Phosphors in der Eiweissfraktion berechnet wurden, sind der überwiegende Teil RNS, die aus demselben Extrakt direkt bestimmt wurden. Für das RNS : DNS-Verhältnis ergibt sich der recht hohe Wert von 3,4. Dies ist besonders auffällig im Vergleich zur Retina

VOL. 4 1960

der Wirbeltiere, in der bei etwa gleicher RNS-Konzentration die zehnfache Menge von DNS—bezogen auf die GTS—vorhanden ist. Von dem leicht säurelöslichen Phosphor liegt fast ein Drittel als freier anorganischer Phosphor vor, ein weiteres Drittel ist durch 7 Min Hydrolyse in n HCl abspaltbar. Dieser 7-Min-Phosphor wird als ein Mass für den Gehalt an energiereichen Phosphorverbindungen verwendet. Insgesamt verteilt sich der Gesamt-Phosphor ungefähr mit folgenden Anteilen auf die Fraktionen: etwa 10% freier anorganischer P, knapp 25% organisch gebundener P in den leicht säurelöslichen Verbindungen, 40–45% Phosphatid-P, 20–25% Eiweiss-P; hiervon der grösste Teil (ca. 18%) in den Nucleinsäuren.

Die Kohlenhydrate (gesamter Zucker nach erschöpfender Säurehydrolyse, als Glucose bestimmt) sind im Vergleich zu den Nerven- und Sinnesgeweben von Wirbeltieren in sehr grosser Menge vorhanden. Daran haben die Reserve-Kohlenhydrate, die im wesentlichen als Glykogen vorliegen, einen erheblichen Anteil (etwa 50% des Gesamt-Zuckers). Der übrige Teil der Kohlenhydrate wird—neben einer geringen Menge von fest in Chitin und Eiweiss gebundenen Zuckern—wohl hauptsächlich von Trehalose gebildet. Diese wird gemeinsam mit den wenig vorhandenen reduzierenden Monosacchariden als Fraktion des freien Zuckers bestimmt (LANGER, 1959). Während der freie Zucker in den Augen von Männchen und Weibchen in gleicher relativer Menge vorliegt, ist die Konzentration an Reserve-Kohlenhydraten sehr verschieden gross. Bezogen auf die GTS sind in den Augen der Männchen nur etwa halb so viel Reserve-Kohlenhydrate enthalten wie in denen der Weibchen. Der Anteil der Reserve-Kohlenhydrate beeinflusst die Grösse der Gesamt-Zuckerwerte so stark, dass für diese ebenfalls kein Mittelwert über beide Geschlechter berechnet werden kann.

Um Veränderungen im Chemismus des Auges in Abhängigkeit von der adäquaten Reizung aufzufinden, wurden die lebenden Tiere verschiedenen Lichteinwirkungen ausgesetzt. Da das Auge von *Calliphora* eine hohe Empfindlichkeit vor allem im UV aufweist (WALTHER und DODT, 1959), wurde in den Versuchen sowohl sichtbares als auch UV-Licht verwendet. Zum Vergleich dienten Tiere, die einem Tag-Nacht-Rhythmus der Beleuchtung gleichmässig während des ganzen Imaginallebens unterlagen, und solche, die als Imagines völlig im Dunkeln lebten. Damit ergeben sich vier verschiedene Intensitätsstufen der Reizwirkung auf das Lichtsinnesorgan:

(1) *Völlige Dunkelheit.* Die Tiere wurden von der Puppe an in absolut dunklem Zuchtraum gehalten; die Entnahme der Tiere und die Präparation der Augen erfolgten bei Rotlicht von Wellenlängen (>660 mμ), für die das *Calliphora*—Auge nahezu unempfindlich ist. Bezeichnung dieser Versuchsgruppe: D.

(2) *Normale Lichteinwirkung.* Die Tiere wurden für die Versuche direkt den üblichen Zuchtbedingungen entnommen, die einen 12-stündigen Hell-Dunkel-Rhythmus aufwiesen. Die Präparation der Augen erfolgte sofort bei schwachem, auf Wärmestrahlung gefiltertem Glühlampenlicht. Diese Tiere dienten als Kontrollgruppe in den meisten Vergleichen; Bezeichnung: Ko.

(3) *Bestrahlung mit sichtbarem Licht.* Die Tiere wurden aus Zuchten wie unter (2) entnommen und 4 Std. lang mit intensivem Glühlampenlicht bestrahlt, das nur die

Wellenlängen zwischen 400 und 800 mμ enthielt. Bezeichnung dieser Versuchsgruppe: S_L.

(4) *Bestrahlung mit ultraviolettem Licht.* Die Tiere wurden aus Zuchten wie unter (2) entnommen und 6 Std. lang mit UV-Licht von geringer Intensität bestrahlt, das mit schwachen Anteilen bis ins Blau reichte. Bezeichnung dieser Versuchsgruppe: S_{uv}.

Bei den Gruppen S_L und S_{uv} standen den Tieren während der Bestrahlungszeit Wasser und Rohrzucker in beliebiger Menge zur Verfügung. Nach Ende der Bestrahlung wurden die Augen sofort präpariert. Die Präparationsbedingungen waren die gleichen wie bei den Tieren der Gruppe Ko.

Um eine deutliche Wirkung zu erhalten, wurden Bestrahlungen von langer Dauer verwendet, die aber von den Tieren gut vertragen wurden. Die Fliegen verhielten sich—bei erhöhter Aktivität—völlig normal; wurden sie nach Ende der Bestrahlung wieder unter normale Zuchtbedingungen gebracht, so blieben alle Tiere am Leben und zeigten keine Schädigung.

Die willkürlich erscheinende Unterteilung der Versuche nach Einwirkung von sichtbarem und ultraviolettem Licht ist gerechtfertigt auf Grund der Eigenschaften der im *Calliphora*-Auge vorhandenen Pigmente. Diese weisen ein Absorptionsspektrum auf (SCHWINCK, 1956), das eine erhebliche Verminderung der Intensität des sichtbaren Lichtes erwarten lässt, bevor dieses an die Sehelemente gelangt, während das UV-Licht durch diese Pigmente wesentlich weniger gemindert zu den Rhabdomen durchdringen kann.

VOL. 4 1960

Über die bei der Bestrahlung wirksame Intensität des Lichtes bezw. die während der ganzen Zeit auf die Sehelemente einwirkende Energiemenge können keine zuverlässigen Angaben gemacht werden. Da die optischen Daten für den dioptrischen Apparat des Facettenauges völlig unbekannt sind, ist es unmöglich, den zu den Rhabdomen gelangenden und dort absorbierten Anteil der eingestrahlten Energie auch nur näherungsweise anzugeben. Nur dieser Teil ist jedoch als Reiz wirksam und könnte für quantitative Vergleiche—z. B. von S_L und S_{uv}—verwendet werden. Deshalb wird auf alle Intensitätsangaben verzichtet; es kann nur vergleichend festgestellt werden, dass die Strahlungsintensität der verwendeten Glühlampe (durch die eine Helligkeit am Bestrahlungsort erzielt wurde, die etwa der Mittagshelligkeit des Tageslichtes entspricht) wesentlich grösser war als die des Quecksilberbrenners. Vergleichende Messungen mit der Thermosäule zeigten ein Verhältnis der am Ort der Tiere einfallenden Gesamt-Strahlungsenergie von etwa 10 : 1. Die geringe UV-Intensität wurde verwendet, weil im Tageslicht der UV-Anteil nur recht klein ist und die Bestrahlungsbedingungen im physiologischen Bereich liegen sollten.

In den Versuchen wurden stets Gruppen von Tieren, die zwei verschiedenen Stufen der Reizintensität ausgesetzt waren, paarweise miteinander verglichen. Analytische Bestimmungen unter Verwendung aller vier Intensitätsstufen wurden nur an einigen Gruppen von Substanzen durchgeführt, und zwar für den säureunlöslichen Stickstoff und die durch Messung des leicht säurelöslichen Phosphors und seiner Fraktionen erfassten Verbindungen. Nachdem sich dabei eine gleichartige Wirkung von sichtbarem und ultraviolettem Licht gezeigt hatte, die lediglich für das UV quantitativ grösser war als für das sichtbare Licht, wurde sie nur noch

an einer weiteren Stelle—beim freien Zucker—getestet. In der Mehrzahl der Versuche wurde dann nur noch der Vergleich zwischen den Gruppen Ko und S_{uv} ausgeführt.

Die Verwendung von UV als Reizlicht hat noch einen wesentlichen Vorteil. Bei der Einstrahlung von sichtbarem Licht ist eine Erwärmung des Auges nicht ausgeschlossen; die Absorption in den Pigmenten macht zumindest eine lokale Temperatur-Erhöhung wahrscheinlich, deren Auswirkungen auf den Stoffwechsel nicht übersehbar sind. Dagegen ist eine Erwärmung durch das in geringer Dosis eingestrahlte UV, das kaum absorbiert wird, nicht zu erwarten. Die Temperatur in den Augen während der Bestrahlung wurde durch Messung mittels Mikro-Thermoelementen (ϕ etwa 0,1 mm) innerhalb der Retina an überlebenden isolierten Köpfen geprüft. Dabei zeigte sich, dass die Temperatur des Auges auch nach stundenlanger UV-Bestrahlung um nicht mehr als $\pm 1°$ von der Umgebungstemperatur abwich.

In vergleichenden Versuchen stellte sich heraus, dass der Fütterungszustand der Tiere und die Tageszeit der Bestimmungen bei den meisten Stoffen keinen Einfluss auf die gefundenen Mengen haben. Es ist also gleichgültig, zu welchem Zeitpunkt des Tag–Nacht–Rhythmus die Tiere aus den Zuchtkästen in den Bestrahlungsversuch genommen werden; nur bei den Zuckerverbindungen besteht eine Abhängigkeit vom Fütterungszustand, so dass tagsüber die Werte von Tier zu Tier recht verschieden sein können. Um ein möglichst einheitliches Ausgangsmaterial für die Versuche zu haben, wurden die Tiere morgens kurz vor Beginn der Hellperiode bei sehr geringem diffusem Licht den Zuchten entnommen und sofort zur Bestrahlung gebracht. Da die Fliegen während Dunkelheit keine nennenswerte Aktivität zeigen und kaum Nahrung aufnehmen, müssen die Unterschiede dann am geringsten sein. Trotzdem ergaben sich bei den Kohlenhydraten grosse Streuungen in den Analysendaten.

Die Versuche wurden zunächst an Männchen und Weibchen in einem Ansatz, später an Gruppen von Tieren durchgeführt, die nach Geschlechtern getrennt waren. Durch den Bezug auf den säureunlöslichen Stickstoff sind diese Versuche ohne weiteres vergleichbar. Jedoch musste zunächst noch geprüft werden, ob Veränderungen der Menge des säureunlöslichen Stickstoffs infolge der Lichteinwirkung auftreten. Dabei wurde noch die Anzahl der Augen als Bezugswert verwendet; die Versuche müssen deshalb nach Männchen und Weibchen getrennt aufgeführt werden. Die Ergebnisse gibt Tab. 2 wieder; es zeigt sich, dass keine Unterschiede zwischen den verschiedenen Versuchsgruppen vorhanden sind. Die zahlenmässige Berechnung erfolgt hier und in allen folgenden Experimenten in der Weise, dass Analysenwerte eines Paares von Versuchsgruppen mit verschiedener Lichteinwirkung prozentual zueinander berechnet werden, und zwar der Wert für die Augen mit stärkerer Reizbelastung in Prozenten dessen für die Augen mit geringerer. Über die Prozentwerte innerhalb einer Versuchsserie wird dann gemittelt; ein auftretender Unterschied des Mittelwertes gegenüber 100% wird mit der Nullhypothese (PÄTAU, 1943) statistisch auf seine Sicherung geprüft. Diese Mittelwerte für den säureunlöslichen Stickstoff sind in die Tab. 3 mit aufgenommen.

Solche Versuchsserien wurden für eine ganze Reihe von Stoffgruppen durchgeführt; bei einer Übersicht stellte sich heraus, dass die Bestrahlungswirkung auf Bau- und Betriebsstoffe der Zelle (im Sinne der Unterteilung der klassischen

TABELLE 2—MENGE DES SÄUREUNLÖSLICHEN STICKSTOFFS IN DEN AUGEN VON TIEREN UNTER VERSCHIEDENEN BELICHTUNGSBEDINGUNGEN.

(Angaben in γ/Auge)

Alter (Tage)	Männliche Tiere N-Gehalt	N-Gehalt	Weibliche Tiere N-Gehalt	N-Gehalt	%
Vergleich: D–Ko:	D	Ko	D	Ko	Ko von D
1	25,0	22,5	20,8	18,6	89
2	—	—	24,6	19,0	77
9	22,5	25,3	18,8	21,1	112
14	32,5	31,7	27,1	26,4	98
25	20,4	30,3	—	—	148
				Mittel:	105
Vergleich: Ko–S_L:	Ko	S_L	Ko	S_L	S_L von Ko
5	—	—	18,0	17,0	95
8	22,3	22,5	—	—	101
10	22,4	22,4	—	—	100
13	—	—	18,7	17,8	95
18	22,5	22,0	—	—	98
23	—	—	18,2	17,9	98
				Mittel:	98
Vergleich: Ko–S_{uv}:	Ko	S_{uv}	Ko	S_{uv}	S_{uv} von Ko
1	22,3	23,2	18,6	19,4	104
8	22,6	22,5	—	—	100
8	20,7	21,8	—	—	105
9	—	—	21,8	19,9	91
12	—	—	18,1	18,3	101
14	31,7	30,4	26,4	25,3	96
16	23,2	22,8	—	—	98
21	—	—	18,2	19,0	104
23	21,8	22,8	—	—	103
				Mittel:	100
Vergleich: D–S_{uv}:	D	S_{uv}	D	S_{uv}	S_{uv} von D
1	25,0	23,2	20,8	19,4	93
14	32,5	30,4	27,1	25,3	94
17	29,9	30,5	24,9	25,4	102
28	25,0	25,7	20,8	21,4	103
				Mittel:	98

Stoffwechselphysiologie) verschieden ist. Deshalb sollen diese getrennt besprochen werden.

Baustoffe. Die Untersuchung erstreckte sich—neben dem säureunlöslichen Stickstoff—vor allem auf die durch Phosphorbestimmung in den Fraktionen nach

SCHNEIDER (1945) erfassbaren Substanzgruppen und auf die Nucleotide. Bei den Nucleinsäuren wurde sowohl die Gesamtmenge durch die entsprechende Phosphorfraktion als auch die RNS direkt bestimmt.

TABELLE 3—ÜBERSICHT ÜBER DIE RELATIVEN WERTE DES GEHALTS AN EINIGEN BAUSTOFFEN IN DEN AUGEN NACH HALTUNG DER TIERE UNTER VERSCHIEDENEN BELICHTUNGSBEDINGUNGEN

Substanz	Vergleich	Anzahl der Versuche	Grenzen %	Mittelwert %
Säureunlöslicher N	D – Ko	5	77 ... 148	105
	Ko – S_L	6	95 ... 101	98
	Ko – S_{uv}	9	91 ... 105	100
	D – S_{uv}	4	93 ... 103	98
Gesamter leicht säurelöslicher P	D – Ko	5	86 ... 108	96
	Ko – S_L	4	92 ... 107	101
	Ko – S_{uv}	11	91 ... 115	100
	D – S_{uv}	4	92 ... 102	98
Lipoid–P	D – Ko	5	92 ... 99	97
	Ko – S_{uv}	9	76 ... 110	100
	D – S_{uv}	3	75 ... 105	94
Gesamter Eiweiss-P	D – Ko	5	91 ... 109	97
	Ko – S_{uv}	6	92 ... 133	104
	D – S_{uv}	4	89 ... 122	102
Heiss–TCA–löslicher Eiweiss–P	Ko – S_{uv}	6	95 ... 107	100
Heiss–TCA–unlöslicher Eiweiss-P	Ko – S_{uv}	4	96 ... 98	97
Ribonucleinsäuren	Ko – S_{uv}	6	93 ... 103	99
Freie Nucleotide	Ko – S_{uv}	6	79 ... 102	92

Für die 8 Baustoffe wurden 18 Serien von Vergleichsversuchen durchgeführt, und zwar für jede Substanz bis zu 4 Kombinationen der Lichteinwirkung in paarweisen Vergleichen. Jede Versuchsserie bestand durchschnittlich aus 6 Einzelversuchen an Tieren von verschiedenem Alter. Da die Stoffe zum grossen Teil Mengenveränderungen aufweisen, die in systematischer Weise vom Imaginalalter der Fliegen abhängen (LANGER, 1960), ist es nicht möglich, die gewonnenen Ergebnisse in Form der Mittelwerte für den Substanzgehalt anzugeben. Die prozentualen Ergebnisse nach der auf S. 290 angegebenen Berechnung sind jedoch für alle Altersstadien annähernd gleich; deshalb wird—um die Tabellen nicht mit den Werten der Einzelversuche zu belasten*—nur der Mittelwert über diese Prozentzahlen und deren Grenzen innerhalb der Versuchsserie angegeben. Die Zusammenstellung für die Baustoffe gibt Tab. 3; darin sind die Vergleichspaare durch die Zusammenstellung der Bezeichnungen der Gruppen (s. S. 288) symbolisiert und die Anzahl der Einzelversuche in jeder Versuchsserie eingetragen.

Bei allen Baustoffen ist kein Einfluss der Belichtung auf die Substanzmengen in den Augen festzustellen. Dies ist besonders wichtig für die Lipoide, deren Menge in der Wirbeltier-Retina sich unter Lichteinwirkung verringert (TAKAMATSU,

* Die Ergebnisse der Einzelversuche sind dargestellt in: H. LANGER, "Chemisch-physiologische Untersuchungen am Auge von *Calliphora erythrocephala* Meig.", Habilitationsschrift an der Naturwissenschaftlichen Fakultät der Universität München, 1959.

1934; TAWARA, 1938; SEKOGUTI, 1953), und für den gesamten leicht säurelöslichen Phosphor. In dieser Fraktion müsste jeder in irgendeiner anderen Fraktion frei werdende Phosphor als Zuwachs erscheinen; sie wird aber weder absolut noch prozentual vermehrt. Weiter ist keine Verminderung bei den Nucleinsäuren festzustellen, wie sie in den retinalen Ganglienzellen des Wirbeltier-Auges gefunden wurde (BRATTGÅRD, 1952). Bei den freien Nucleotiden scheint eine geringe Verminderung durch die Bestrahlung aufzutreten. Jedoch sind bei der verwendeten Analysentechnik die aus der Aufarbeitung von Frischmaterial erhaltenen Absolutwerte für diese Substanzgruppe etwas zu hoch—wie der Vergleich mit Trockenmaterial-Aufarbeitungen gezeigt hat (LANGER, 1960)—, da gelöster Zucker bei der Bestimmung interferiert. Der Zucker aber wird bei Bestrahlung vermindert (vergl. S. 296); die Berechnung ergibt, dass dieser verringerte Störfaktor ziemlich genau die Erniedrigung der Werte ausmacht. Die Menge der Nucleotide ist also wahrscheinlich konstant.

Bei den Baustoffen ist der Vergleich mit den in völliger Dunkelheit aufgewachsenen Tieren von besonderer Bedeutung; obwohl die Augen dieser Tiere den adäquaten Reiz niemals bekommen haben, sind sie in ihrem chemischen Aufbau in völlig normaler Weise ausgebildet. Dies stellt einen Gegensatz zu den für einige Substanzen in der Wirbeltier-Retina erhobenen Befunden (BRATTGÅRD, 1952; HELLSTRÖM und ZETTERSTRÖM, 1956) dar.

Betriebsstoffe. Bei den Betriebsstoffen sind die normalerweise auftretenden Schwankungen in den vorhandenen Mengen viel erheblicher als bei den Baustoffen, sie verlaufen—z. B. im Zusammenhang mit der Nahrungsaufnahme—oft in kurzen Zeiten. Infolgedessen sind für die Versuche die Aufzuchtbedingungen der Tiere weniger wichtig als die Haltungsbedingungen in der letzten Zeit vor dem Ansatz. Deshalb wurden unter den Betriebsstoffen nur bei der Analyse des freien anorganischen und des Hydrolyse-Phosphors die Tiere in Dunkelaufzucht mit betrachtet. Für die Analysen der Kohlenhydrate war es wichtiger, dass die Tiere am Ende der Dunkelperiode den Zuchtkästen entnommen wurden.

Die Versuche für die Betriebsstoffe erfolgten in genau derselben Art paarweise und wurden ebenso prozentual berechnet wie die für die Baustoffe. Für die 5 Betriebsstoffe wurden 12 Serien von Vergleichen mit jeweils durchschnittlich 5 Einzelversuchen durchgeführt. Die Ergebnisse zeigt Tab. 4 in Form der Übersicht über die Mittelwerte in gleicher Weise wie bei den Baustoffen.

Mit der Einwirkung von Licht zeigt der freie anorganische Phosphor eine Erhöhung. Sie ist bei dem Vergleich der dunkel gehaltenen Tiere mit den—helladaptierten—Kontrollen noch gering, wird jedoch bei der Bestrahlung mit UV-Licht deutlich. Im Vergleich der mit sichtbarem Licht bestrahlten Tiere mit den Kontrollen findet sich in den Paaren kein Unterschied. Die Erhöhung der Menge des anorganischen Phosphors beim Vergleich D–S_{uv} ist im Mittel etwa gleich der Summe aus den Erhöhungen bei D–Ko und Ko–S_{uv}, wie dies zu erwarten war.

Der Vermehrung des freien anorganischen Phosphors steht die Konstanz des gesamten leicht säurelöslichen Phosphors gegenüber. Dies bedeutet, dass das

TABELLE 4—ÜBERSICHT ÜBER DIE RELATIVEN WERTE DES GEHALTS AN EINIGEN BETRIEBSSTOFFEN IN DEN AUGEN NACH HALTUNG DER TIERE UNTER VERSCHIEDENEN BELICHTUNGSBEDINGUNGEN

Substanz	Vergleich	Anzahl der Versuche	Grenzen %	Mittelwert %	P-Wert
Freier anorganischer P	D – Ko	5	86 ... 132	106	>0,1
	Ko – S_L	4	92 ... 105	100	
	Ko – S_{uv}	5	101 ... 138	115	<0,005
	D – S_{uv}	4	101 ... 146	122	
Hydrolyse-P nach 7 Min Hydrolyse	D – Ko	5	94 ... 101	96	
	Ko – S_L	4	87 ... 102	94	>0,1
	Ko – S_{uv}	5	86 ... 108	99	
	D – S_{uv}	4	87 ... 116	100	
Gesamt-Zucker nach Hydrolyse	Ko – S_{uv}	6	59 ... 92	71	<0,005
Freier Zucker	Ko – S_L	4	90 ... 138	118	<0,005
	Ko – S_{uv}	6	103 ... 168	133	
Milchsäure	Ko – S_{uv}	6	92 ... 167	117	>0,1

zusätzlich freigewordene Phosphat aus den Verbindungen der TCA-Fraktion selbst kommt, nicht aber aus den Phosphorverbindungen anderer Fraktionen stammen kann.

Zum weiteren Vergleich ist die Fraktion des Hydrolyse-Phosphors wichtig, die ebenso wie der gesamte leicht säurelösliche Phosphor unter allen Bedingungen konstant ist.

Da die leicht säurelöslichen Phosphorverbindungen bereits am dritten Tag des Imaginallebens der Fliegen einen Wert erreichen, der unter normalen Bedingungen bis zum Lebensende gleichbleibt, ist es möglich, in diesem Falle auch über die absoluten Zahlen für den Phosphorgehalt zu mitteln. In Tab. 5 sind die Mittelwerte aus allen Einzelbestimmungen in den 4 Versuchsgruppen der Lichteinwirkung zusammengestellt. Bei konstanten Werten für den leicht säurelöslichen Phosphor zeigt sich im freien anorganischen Phosphor der Anstieg von den Werten für Augen dunkel gehaltener bis zu denen UV-bestrahlter Tiere; für die Gruppen Ko und S_{uv} ist auch die Differenz zwischen den beiden Mittelwerten statistisch gesichert ($P < 0{,}01$). Die Werte für die Augen der mit sichtbarem Licht bestrahlten Tiere liegen höher als die für die Kontrollen, die Lichtbestrahlung hat also einen gewissen Einfluss, der aber gering ist und wohl deshalb beim paarweisen Vergleich der Gruppen nicht deutlich wurde. Beim Hydrolyse-Phosphor wird auch in dieser Zusammenstellung die Konstanz bei allen Bedingungen deutlich. Der Mittelwert der Gruppe Ko stimmt annähernd mit dem Gesamt-Mittel aller Tiere überein; für dieses ist die Streuung kleiner als für die Gruppe der Kontrolltiere allein.

Die Erhöhung der Werte des anorganischen Phosphors bedeutet somit eine entsprechende Verminderung der Differenz Hydrolyse-Phosphor minus freier anorganischer Phosphor (Tab. 5, letzte Spalte); dieser 7-Min-Phosphor verringert sich also mit der Bestrahlung, und zwar z.B. für den Vergleich Ko–S_L um 13%, für den Vergleich D–S_{uv} um 29%.

VOL. 4 1960

TABELLE 5—MITTELWERTE AUS DEN ERGEBNISSEN DER ANALYSEN FÜR DEN LEICHT SÄURELÖSLICHEN PHOSPHOR UND SEINE FRAKTIONEN IN DEN AUGEN VON TIEREN UNTER VERSCHIEDENEN BELICHTUNGSBEDINGUNGEN

(Angaben in γ/mg säureunlöslichen Stickstoff)

Gruppe	Gesamter leicht säurelöslicher P				Freier anorganischer P				Hydrolyse-P nach 7 Min Hydrolyse				7-Min-P
	Anzahl	Grenzen	Mittelwert	σ	Anzahl	Grenzen	Mittelwert	σ	Anzahl	Grenzen	Mittelwert	σ	
D	6	21,9 ... 40,5	28,0		7	5,3 ... 8,6	6,9		7	14,4 ... 18,2	16,5		**9,6**
Ko	51	23,6 ... 39,1	28,9	±3,99	23	4,0 ... 12,3	7,3	±1,92	23	13,7 ... 19,4	16,4	±1,55	**9,2**
S_L	4	23,6 ... 29,0	25,9		4	7,0 ... 10,8	8,5		4	14,5 ... 19,1	16,4		**8,0**
S_{uv}	12	20,6 ... 40,4	30,2		7	7,7 ... 12,8	9,7		7	15,5 ... 18,4	16,9		**6,8**
Mittel:			28,9	±4,73							16,5	±1,44	

Der Gesamt-Zuckergehalt wird bei Bestrahlung erniedrigt. Die aufgefundene Differenz von 29% ist besonders bemerkenswert, weil die zum Vergleich benutzten Kontrolltiere bei der Entnahme aus der Zucht noch keine Nahrung aufgenommen haben und den niedrigsten während des Tages feststellbaren Gesamt-Zuckerwert zeigen, die Versuchstiere aber während der Bestrahlung reichlich gefüttert werden. Der tatsächliche Verbrauch an Zucker in den Augen geht also weit über den Anteil von reichlich einem Viertel der vorhandenen Gesamtmenge hinaus, da mit einer erheblichen Nachlieferung gerechnet werden muss.

Entgegen diesem Befund für den Gesamt-Zucker ist bei dem freien Zucker ein starker Anstieg in den nach Bestrahlung gefundenen Mengen festzustellen. Hier liegt ebenso wie beim freien anorganischen Phosphor der Effekt von Bestrahlung mit sichtbarem und mit UV-Licht in der gleichen Richtung, und er ist ebenfalls bei UV-Einwirkung quantitativ grösser.

Zusammen mit den Ergebnissen für den Gesamt-Zucker resultiert eine sehr starke Abnahme der Reserve-Kohlenhydrate, die auf nahezu ein Drittel der Ausgangsmenge absinken. Sie werden wahrscheinlich nicht sofort vollständig verbraucht, sondern zum Teil nur depolymerisiert und stehen als Oligosaccharide zur Verfügung. Ausserdem kann eine Vermehrung der niedrigmolekularen Zuckeranteile auch von einem verstärkten Antransport herrühren; beides deutet auf eine Vergrösserung der Menge sofort verfügbarer Energieträger hin.

TABELLE 6—EINFLUSS VON 6-STÜNDIGER BESTRAHLUNG MIT ULTRAVIOLETTEM LICHT AUF DEN GEHALT AN KOHLENHYDRATEN IN DEN AUGEN VON NICHT GEFÜTTERTEN TIEREN

(Angaben in γ/mg säureunlöslichen Stickstoff)

Alter (Tage)	Geschl.	Gesamt-Zucker		Freier Zucker		Reserve-Kohlenhydrate	
		Ko	S_{uv}	Ko	S_{uv}	Ko	S_{uv}
13	m	443	0	249	0	167	0
20	w	772	116	230	77	512	<10

Führt man die Bestrahlungsversuche ohne Fütterung der Fliegen durch, so wird die 6-stündige Hungerperiode von den Tieren eben noch vertragen, wenn sie auch in ihrer Aktivität stark nachlassen. Der Gesamt-Zuckergehalt sinkt dann in den Augen rapide ab, und zwar bei den Männchen auf so geringe Mengen, dass sie analytisch nicht mehr erfassbar sind. Bei den Weibchen mit ihrem höheren Gehalt an Reserve-Kohlenhydraten in den Augen wird der Zucker unter denselben Bedingungen nicht restlos verbraucht; es ist interessant, dass selbst unter den extremen Bedingungen eines solchen Hungerversuchs der Spiegel an freiem Zucker noch recht hoch bleibt, während sich die Reserve-Kohlenhydrate auf einen völlig unwesentlichen Rest verringern (Tab. 6).

Im Zusammenhang mit den Zuckerbestimmungen wurden Analysen des Milchsäuregehaltes durchgeführt, um eine möglicherweise erfolgende Glykolyse zu erkennen, deren Auftreten bei Bestrahlung zur Unterstützung der Energieproduktion bei Mangel an Sauerstoff im Augengewebe denkbar wäre. Die Versuche zeigten eine leichte Tendenz zur Erhöhung der Menge nach Bestrahlung, jedoch keine signifikante Differenz. Beim intakten Tier kann also Glykolyse in den Augen, auch bei starker Beanspruchung des Stoffwechsels, keine wesentliche Rolle spielen.

DISKUSSION

Die in die sensiblen Zellen eines Sinnesorgans einfallende spezifische Reizenergie ist wesentlich geringer als die Energie der Erregung, die durch sie ausgelöst wird. Es muss also im Zusammenhang mit der Erregungsbildung Arbeit aufgewendet werden, um nach Entladung der Sinneszelle das Potential wieder aufzubauen. Im Hinblick auf diese Fragen des Energiebedarfs und des Grundstoffwechsels sind an der Retina von Wirbeltieren eine Anzahl von Untersuchungen über den Einfluss der Belichtung durchgeführt worden, die jedoch nicht zu einheitlichen Ergebnissen geführt haben.

VOL. 4 1960

An der isolierten Retina *in vitro* erhielten verschiedene Bearbeiter (OGUCHI, 1934; CAMPOS, 1936a, b; CHASE und SMITH, 1939; HWANG, 1950) keine Unterschiede im Sauerstoffverbrauch zwischen dunkel gehaltener und belichteter bezw. dunkel- und helladaptierter Retina, jedoch fand CAMPOS (1936a, b) an der Retina im Dunkeln eine erhöhte 0_2-Aufnahme, wenn das Auge vorher *in situ* intensiv bestrahlt worden war. Auch nach kurzzeitiger Vorbelichtung der isolierten Retina ergab sich ein erhöhter Atmungswert im Dunkeln (TAKANO, 1935). Bei Belichtungswechsel an der Retina *in vitro* erhielten JONGBLOED und NOYONS (1936) in den zwischen den Belichtungen liegenden Dunkelperioden eine höhere 0_2-Aufnahme und CO_2-Abgabe als im Licht. Auch nach LE-VAN-NHAM (1955) ist der Sauerstoffverbrauch der Retina im Dunkeln grösser als bei Belichtung. In gleicher Richtung liegen Ergebnisse von HANAWA *et al.* (1955) an isolierten Stäbchenaussengliedern, während HUBBARD (1954) keine Unterschiede in der O_2-Aufnahme vor und nach Bleichung einer Suspension von Stäbchenaussengliedern fand.

Ebenso wenig einheitlich sind die Ergebnisse, die über den Phosphatstoffwechsel der Retina erhalten wurden. Die bei Belichtung auftretende Säurebildung soll auf den Austritt von freier Phosphorsäure zurückzuführen sein (LANGE und SIMON, 1922; TAKAMATSU, 1934; OGUCHI, 1938). TAWARA (1938) fand auch in der *in situ* helladaptierten Retina des Kaninchens eine grössere Menge von freiem Orthophosphat als in der dunkeladaptierten. Dies konnte an der Retina hell- und dunkelgehaltener Frösche (SEKOGUTI, 1953) und bei kurzzeitiger Belichtung der isolierten Frosch-Retina (HONJO und KATO, 1952) nicht bestätigt werden, jedoch wurde bei Dunkeladaptation nach Belichtung der isolierten Retina eine erhöhte Phosphataufnahme gefunden (HONJO und KATO, 1953).—Übereinstimmend wird von verschiedenen Autoren eine Verminderung der Menge des Phosphatid-Phosphors in der Wirbeltier-Retina nach Belichtung angegeben (TAKAMATSU, 1934; TAWARA, 1938; SEKOGUTI, 1953).—Bei den Nucleinsäuren zeigten sich nach mikrochemischen Bestimmungen (EHRLICH und DISCHE, 1950) keine Differenzen zwischen den Retinae von hell- und dunkeladaptierten sowie intensiv bestrahlten Säugetieren. Dagegen fand BRATTGÅRD (1952) mittels Histospektrophotometrie Unterschiede im RNS-Gehalt von Ganglienzellen in der Retina in Abhängigkeit von der Belichtung. —Insgesamt ist ein erhöhter Umsatz

bei den Phosphorverbindungen in der Wirbeltier-Retina als Folge der Belichtung zu vermuten; die Ergebnisse der Untersuchungen mit kurzzeitiger Belichtung (HONJO und KATO, 1952, 1953, 1955) machen es jedoch unwahrscheinlich, dass es sich dabei um Vorgänge handelt, die mit den Primärprozessen in direkter Verbindung stehen. Die Erhöhung der Menge des freien anorganischen Phosphats und die Verringerung des Pyrophosphats und des Phosphagens der Retina nach langdauernder Belichtung *in situ* (TAWARA, 1938) könnten aber auf einen vermehrten Verbrauch von energiereichen Phosphorverbindungen hinweisen.

Im Hinblick auf den Kohlenhydratstoffwechsel wurden von MÜLLER (1926) histochemische Untersuchungen über den Glykogengehalt der Retina des Frosches durchgeführt. Nach kurzzeitiger Belichtung eines Auges (Dauer einige Minuten) ergab sich keine Veränderung in der Menge des Glykogens. Dagegen konnte GOURÉVITCH (1951a, b) mit quantitativ-analytischer Methodik zeigen, dass beim Karpfen nach langdauernder Belichtung in der Retina des belichteten Auges weniger Glykogen vorhanden ist als im dunkelgehaltenen Auge desselben Tieres. Dieses Ergebnis liess sich auch histochemisch an der Retina des Goldfischs erhalten (GOURÉVITCH, 1954).

Am Facettenauge sind bisher nur wenige Stoffwechseluntersuchungen durchgeführt worden. Zuerst wurde von BRAMMERTZ (1915) bei histologischen Untersuchungen über den Glykogengehalt von Sehzellen nachgewiesen, dass bei der Stubenfliege (*Musca domestica*) nach längerer Dunkelhaltung mehr Glykogen in der Retinula vorhanden ist als bei Tieren in normaler Helligkeit. Bestrahlungsversuche mit 6 und 24 Std. Lichteinwirkung zeigten eine weitere beträchtliche Abnahme der Glykogenmenge. BRAMMERTZ schliesst daraus, dass das Glykogen 'in irgendeinem ursächlichen Zusammenhang mit der Belichtung steht'. Entsprechend fand GOURÉVITCH (1951c) am Auge der Languste (*Palinurus vulgaris*), dass sich das Glykogen in den Sehzellen bei Belichtung vermindert; nach 24 Std. waren fast keine Glykogengranula in den Augen mehr nachweisbar.—Von AUTRUM (unveröff. Versuche) wurden an isolierten Augen von *Calliphora* Bestimmungen des Sauerstoffverbrauchs bei Wechsel von Dunkelheit und Bestrahlung mit sichtbarem Licht durchgeführt; dabei zeigte sich während der Belichtung eine gegenüber den Dunkelperioden vermehrte 0_2-Aufnahme.

In der vorliegenden Arbeit wurde die Wirkung von langdauernder Bestrahlung auf den Chemismus des Auges untersucht, dabei wurde sowohl sichtbares, als auch UV-Licht zur Reizung verwendet. Ultraviolettes Licht wird von den Insekten ebenso als Reiz wahrgenommen wie das für Wirbeltiere sichtbare Licht (KÜHN, 1927; BERTHOLF, 1931, 1933; DAUMER, 1956; WALTHER und DODT, 1957, 1959); das nahe UV hat sogar die grösste relative Reizwirksamkeit. Der sichtbare Spektralbereich reicht bei Dipteren bis ins kurzwellige UV (BERTHOLF, 1933, WALTHER und DODT, 1959), wahrscheinlich liegt die Grenze unterhalb 250 mμ. Es darf also angenommen werden, dass die Bestrahlung mit einem schwachen Licht zwischen 254 und 436 mμ eine adäquate Reizung darstellt. Während beim UV grosse Lichtstärken vermieden wurden, um nicht mit unphysiologischer Dosis zu bestrahlen, wurden bei den Experimenten mit sichtbarem Licht hohe Intensitäten verwendet, die etwa der Stärke des Himmelslichtes entsprachen.

Die durchgeführten Versuche können dazu dienen, Hinweise auf die Stoffgruppen zu erhalten, in denen sich Veränderungen als Folge der Bestrahlung

ergeben. Alle untersuchten Baustoffe, also die Substanzen, die im wesentlichen die Struktur des Organs ausmachen—vor allem Eiweisse, Lipoide, Nucleinsäuren, Chitin—, zeigen keine Veränderungen der Mengen infolge der Lichteinwirkung. Dieses Ergebnis kann einerseits darauf zurückgeführt werden, dass ein Teil der Substanzen nicht am funktionsabhängigen Stoffwechsel beteiligt ist; dies ist für das Chitin wahrscheinlich und bei manchen Eiweissen und den DNS möglich. Andererseits besteht die Möglichkeit, dass Umsetzungen innerhalb der Stoffgruppe (z.B. der Phosphatide) stattfinden oder die veränderten oder verbrauchten Stoffe stets in gleicher Menge vom laufenden Bildungsstoffwechsel ersetzt werden. So wurde an der Wirbeltier-Retina gezeigt (HONJO und KATO, 1952, 1955; SEKOGUTI, 1953), dass sich die Grösse der Umsatzrate in der Phosphorproteid-Fraktion in Abhängigkeit von der Lichteinwirkung ändert.

Alle bei den Bestriebsstoffen festgestellten Veränderungen deuten auf einen vermehrten Verbrauch bei diesen Verbindungen hin. Dies zeigt sich sowohl in der Verminderung des Gesamt-Zuckers als auch in der Vermehrung des freien Zuckers, die sowohl durch Depolymerisation von Reserve-Kohlenhydraten als auch durch Nachlieferung von Oligosacchariden zustande kommen kann. Beides muss als Aktivierung von Energiereserven für baldige Verwendung gedeutet werden. Ein erhöhter Energieverbrauch spricht auch aus der Verringerung der Menge des 7-Min-Phosphors. Da die energiereichen Phosphorverbindungen laufend auf Kosten der Kohlenhydrate nachgebildet werden, sind die erniedrigten Mengenwerte kein Mass für den Verbrauch. Es bleibt offen, ob sie durch unzureichende Neubildung entstehen oder als ein auf den Energieverbrauch eingestellter Spiegel aufzufassen sind.

VOL. 4 1960

Auch bei den Kohlenhydraten ist der Verbrauch sicher viel grösser als aus der gefundenen Verringerung der Gesamt-Zuckermenge hervorgeht. Denn bei erhöhtem Verbrauch werden gewiss aus dem Körper des Tieres Stoffe in vermehrtem Umfang zu den Augen hintransportiert. Dazu kommt, dass sich die Fliegen im Dunkeln wenig bewegen und kaum Nahrung aufnehmen, während sie im Licht aktiv sind, gefüttert werden müssen und reichlich fressen. Wenn aber doch eine Verminderung der Mengen der Betriebsstoffe aufgefunden wurde, so zeigt dies, dass eine sehr erhebliche Vergrösserung in deren Umsatz bei Belichtung auftreten muss. Darauf geben auch die Versuche mit hungernden Tieren einen Hinweis; eine quantitative Auswertung ist aber nicht möglich, da die Aktivität der bestrahlten Tiere nicht ausgeschaltet werden kann.

Die dargestellten Versuche weisen auf einen erhöhten Energiebedarf des Auges von Calliphora *bei seiner Funktion hin; der energieerzeugende Grundstoffwechsel wird während der Belichtung erheblich vermehrt.* Wie aus den Vergleichsversuchen hervorgeht, ist die Wirkung von sichtbarem und UV-Licht dabei gleichartig. Aus den erhaltenen Werten ist jedoch zu entnehmen, dass die Bestrahlung mit UV wesentlich stärker wirksam ist als die mit sichtbarem Licht; obwohl die eingestrahlte Energie beim sichtbaren Licht viel grösser war, liegen die Veränderungen niedriger als beim UV.

Damit deutet sich auch bei der Wirkung auf den Stoffwechsel der Unterschied in der Stärke der Reizwirksamkeit von verschiedenen Spektrallichtern an. Dies dürfte mindestens teilweise in der Pigmentierung des Auges begründet sein. Die Pigmente im *Calliphora*-Auge (SCHWINCK, 1956) sind für UV sehr viel besser durchlässig als für sichtbares Licht. Elektrophysiologische Versuche, in denen die pigmentarme Mutante white-apricot mit der Normalform von *Calliphora* verglichen wurde, zeigten, dass die viel geringere Empfindlichkeit der Augen der +—Tiere im sichtbaren Bereich vor allem von deren Pigmentierung herrührt (AUTRUM, 1955).

Aus den bisherigen Experimenten ist über die Grösse der Lichtwirksamkeit keine Aussage zu machen. Auch die Erhöhung des Grundstoffwechsels ist wegen der am intakten Tier nicht übersehbaren Stoffnachlieferungen bisher nicht quantitativ zu fassen. Jedoch sind hierfür experimentelle Möglichkeiten durch Ausschaltung der entsprechenden Faktoren gegeben; über derartige Versuche wird an anderer Stelle berichtet werden.

Die erhaltenen Ergebnisse zeigen—in Übereinstimmung mit den Sauerstoffverbrauchs-Messungen von AUTRUM (s. Seite 298)—für das Auge von *Calliphora* ein völlig anderes Verhalten des Stoffwechsels, als es an der Retina der Wirbeltiere gefunden wurde. Bei diesen war die Tendenz festzustellen, dass der Stoffwechsel in der Dunkelheit nach voraufgehender Lichteinwirkung höher ist als im Licht, was häufig mit den Erscheinungen der Adaptation in Zusammenhang gebracht worden ist (z.B. JONGBLOED und NOYONS, 1936; CAMPOS, 1936b); vielleicht besteht für die Restitution nach der Reizung ein erhöhter Energiebedarf. Dagegen scheint beim *Calliphora*-Auge ein solcher Bedarf an Energie nach Ende der Lichteinwirkung nicht vorhanden zu sein. Vielmehr zeigt es ähnliche Verhältnisse, wie sie vom Zentralnervensystem der Vertebraten her bekannt sind (Zusammenfassung MCILWAIN, 1959); hier wie dort sind die Sauerstoffaufnahme und der Zuckerverbrauch zu der Zeit gesteigert, zu der die Erregung erfolgt. Die Gemeinsamkeiten beider Gewebe liegen darin, dass die Funktion bei grosser Geschwindigkeit der Reizverarbeitung und fehlender Adaptation erfolgt. Damit diese im Auge von *Calliphora* praktisch augenblicklich geschehen kann (AUTRUM, 1950), wird die in der Wirbeltier-Retina später für die Restitution aufgewendete Energie vielleicht für Vorgänge während der Reizeinwirkung ausgegeben, die den völligen Abbau der photolabilen Substanzen verhindern und diese in einem Zwischenzustand (AUTRUM, 1952) halten, aus dem sie nach dem Ende der Reizung schnell wieder in den Ausgangszustand gebracht werden. Hierin läge ein weiterer während der Belichtung ablaufender energieverbrauchender Prozess vor.

Damit wird der zusätzliche Bedarf an Energie im Auge verständlich; die energieverbrauchenden Vorgänge laufen sämtlich während der Dauer der Reizung und in unmittelbarem Anschluss an sie ab; eine Nachwirkung besteht nicht. So drückt sich wohl auch im Stoffwechselgeschehen der Unterschied des schnell reagierenden Facettenauges gegenüber dem Linsenauge aus, das eine langdauernde Nachwirkung der eingetroffenen Reize aufweist. Trotz der wahrscheinlich gleichartigen photolabilen Grundsubstanz sind die Mechanismen der Reizverarbeitung bei beiden Augentypen offenbar sehr verschieden.

VOL. 4 1960

ZUSAMMENFASSUNG

(1) Es wird eine Übersicht über den chemischen Aufbau des Auges von *Calliphora* gegeben (Tab. 1); auffallend sind die geringe DNS-Menge(RNS:DNS-Verhältnis ist 3,4 : 1) und der hohe Gehalt an Kohlenhydraten, insbesondere Reserve-Kohlenhydraten in Form von Glykogen.

(2) Mehrstündige kontinuierliche Bestrahlung der lebenden Tiere mit sichtbarem und ultraviolettem Licht hat keinen Einfluss auf den Gehalt der Augen an allen untersuchten Baustoffen (z.B. Gesamt-Eiweiss, Nucleinsäuren, Phosphatiden).

(3) Dieselben Bestrahlungen ergeben Veränderungen im Gehalt an Betriebsstoffen, die auf eine Aktivierung von Reserven und eine vermehrte Energieproduktion hindeuten. Es zeigen sich Verminderung der Menge der energiereichen Phosphorverbindungen und der Gesamt-Kohlenhydrate—vor allem des Glykogens—und gleichzeitige Erhöhung des Spiegels der freien Zucker. Sichtbares und UV-Licht wirken in gleicher Weise, aber mit unterschiedlicher Intensität; schwache UV-Bestrahlung ist stärker wirksam als kräftige mit sichtbarem Licht.

(4) Haltung der Imagines (von der Puppe an) in völliger Dunkelheit bewirkt keine Veränderung im chemischen Aufbau der Augen; es ist eine völlige Übereinstimmung der Ausbildung mit den unter Normalbedingungen gehaltenen Fliegen festzustellen.

(5) Aus den Ergebnissen wird geschlossen, dass die Intensivierung des energieerzeugenden Grundstoffwechsels im Facettenauge von *Calliphora* während der Belichtung eine funktionsspezifische Erscheinung in Zusammenhang mit der Reizverarbeitung und der Erregungsbildung des Sinnesorgans darstellt.

LITERATUR

AUTRUM H. (1950) Die Belichtungspotentiale und das Sehen der Insekten. *Z. vergl. Physiol.* **32**, 176.

AUTRUM H. (1952) Über zeitliches Auflösungsvermögen und Primärvorgänge im Insektenauge. *Naturwissenschaften* **39**, 290.

AUTRUM H. (1955) Die spektrale Empfindlichkeit der Augenmutation white-apricot von *Calliphora erythrocephala*. *Biol. Zbl.* **74**, 515.

AUTRUM H. und STUMPF H. (1953) Elektrophysiologische Untersuchungen über das Farbensehen von *Calliphora*. *Z. vergl. Physiol.* **35**, 71.

BERTHOLF L. (1931) The distribution of stimulation efficiency in ultra-violet spectrum for the honeybee. *J. agric. Res.* **43**, 703.

BERTHOLF L. (1933) The extent of the spectrum for *Drosophila* and the distribution of stimulative efficiency in it. *Z. vergl. Physiol.* **18**, 32.

BRAMMERTZ W. (1915) Über das normale Vorkommen von Glykogen in der Retina. *Arch. mikr. Anat.* **86**, 1.

BRATTGÅRD S.-O. (1952) The importance of adequate stimulation for the chemical composition of retinal ganglion cells during early post-natal development. *Acta radiol., Stockh.* Suppl. **96**.

BRINDLEY G. S. (1958) Physiology of vision. *Annu. Rev. Physiol.* **20**, 559.

CAMPOS R. (1936a) L'azione della luce sulla respirazione e sulla glicolisi della retina. *Boll. Soc. ital. Biol. sper.* **11**, 320.

CAMPOS R. (1936b) Ricerche sul ricambio della retina. *Ann. Ottalm.* **64**, 456, 538, 577.

CHASE A. M. und SMITH E. L. (1939) Regeneration of visual purple in solution. *J. gen. Physiol.* **23**, 21.

COLLINS F. D. (1954) The chemistry of vision. *Biol. Rev.* **29**, 453.

DAUMER K. (1956) Reizmetrische Untersuchungen über das Farbensehen der Bienen. *Z. vergl. Physiol.* **38**, 413.

EHRLICH G. und DISCHE Z. (1950) Content of DNA and RNA of retina under various conditions. *Proc. Soc. exp. Biol., N.Y.* **74**, 40.

FISKE G. H. und SUBBAROW Y. (1925) The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

FUJITA A. und IWATAKE D. (1931) Bestimmung des echten Blutzuckers ohne Hefe. *Biochem. Z.* **242**, 43.

GOLDSMITH T. H. (1958a) On the visual system of the bee (*Apis mellifera*). *Ann. N.Y. Acad. Sci.* **74**, 223.

GOLDSMITH T. H. (1958b) The visual system of the honey bee. *Proc. nat. Acad. Sci., Wash.* **44**, 123.

GOURÉVITCH A. (1951a) Recherches sur la consommation du glycogène par la rétine à la lumière. *C. R. Soc. Biol., Paris* **145**, 232.

GOURÉVITCH A. (1951b) Consommation du glycogène par la rétina à la lumière et à l'obscurité. *J. Physiol. Path. gén.* **43**, 255.

GOURÉVITCH A. (1951c) La glycogènolyse dans les cellules visuelles de l'œil éclairé de la langouste. *C. R. Soc. Biol., Paris* **145**, 1839.

GOURÉVITCH A. (1954) La localisation histologique du glycogène dans la rétine des poissons et sa consommation à la lumière. *J. Physiol. Path. gén.* **46**, 633.

HANAWA J., KIMURA E., und HOSOYA Y. (1955) The respiration of the isolated outer segments of rods. *Jap. J. Physiol.* **5**, 322.

HELLSTRÖM B. und ZETTERSTRÖM B. (1956) The effect of light on the manifestation of the electroretinogram and on the histochemically demonstrable SH-groups in the retina. *Exp. Cell Res.* **10**, 248.

HOFFMANN C. (1960) Belichtungspotentiale der Insekten und Sauerstoffdruck. *Verh. dtsch. zool. Ges.* 1959, **53**, 224.

HONJO I. und KATO M. (1952) Bildung und Ausscheidung der Phosphorsäure von der Froschretina. *Seiro-Seitai* **5**, 43.

HONJO I. und KATO M. (1953) On the diffusion of ^{32}P from rod outer segments. *Annot. zool. Jap.* **26**, 186.

HONJO I. und KATO M. (1955) Aufnahme des Radiophosphors durch die Froschretina. *Z. vergl. Physiol.* **37**, 169.

HORWITT N. B. (1952) Determination of inorganic serum phosphorus by means of stannous chloride. *J. biol. Chem.* **199**, 537.

HUBBARD R. (1954) The respiration of the isolated rod outer limb of the frog retina. *J. gen. Physiol.* **37**, 373.

HULLIN R. P. und NOBLE R. L. (1953) The determination of lactic acid in microgram quantities. *Biochem. J.* **55**, 289.

HWANG T. (1950) Respiration of retinal tissue. *Jap. J. Physiol.* **1**, 169.

JONGBLOED J. und NOYONS A. K. (1936) Sauerstoffverbrauch und Kohlendioxydproduktion der Froschretina bei Dunkelheit und bei Licht. *Z. Biol.* **97**, 399.

KAMPA E. M. (1955) Euphausiopsin, a new photosensitive pigment from the eyes of euphausiid crustaceans. *Nature, Lond.* **175**, 996.

KÜHN A. (1927) Über den Farbensinn der Bienen. *Z. vergl. Physiol.* **5**, 762.

LANGE H. und SIMON M. (1922) Über Phosphorsäureausscheidung der Netzhaut bei Belichtung. *Hoppe-Seyl. Z.* **120**, 1.

LANGER H. (1956) Zur photometrischen Mikrobestimmung von Riboseverbindungen, insbesondere RNS. *Naturwissenschaften* **43**, 19.

LANGER H. (1959) Zur Bestimmung einer Fraktion des "freien Zuckers" in Insektengewebe. *Z. Naturf.* **14b**, 480.

LANGER H. (1960) Über den chemischen Aufbau des Facettenauges von *Calliphora erythrocephala* Meig. und seine Veränderungen mit dem Imaginalalter. *Z. vergl. Physiol.* **42**, 595.

LE-VAN-NHAM (1955) La consommation d'oxygène de la rétine de bœuf à la lumière de jour et dans l'obscurité. *Bull. Soc. zool. Fr.* **80**, 70.

MEJBAUM W. (1939) Über die Bestimmung kleinster Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Hoppe-Seyl. Z.* **258**, 117.

MEYERHOF O. (1931) Über den Kohlenhydratverbrauch bei der aeroben Tätigkeit des Kaltblütermuskels. *Biochem. Z.* **237**, 427.

MCILWAIN H. (1959) *Biochemistry and the Central Nervous System*, 2. Aufl. Little, Boston.

MÜLLER C. (1926) Das Glykogen der Retina des Frosches. *Z. ges. Anat.*, I. *Z. Anat. Entw.-Gesch.* **81**, 220.

NELSON N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375.

OGUCHI T. (1934) Über den Stoffwechsel der Netzhaut—VII. Über Veränderungen des Stoffwechsels bei Belichtung der Dunkelfroschnetzhaut. *Acta Soc. ophthal. jap.* **38**, 99.

OGUCHI T. (1938) Experimentelle Studien über den Einfluss der verschiedenartigen Bedingungen auf den Stoffwechsel der Netzhaut—V. Über die Veränderungen des Stoffwechsels der Netzhaut bei Belichtung. *Acta Soc. ophthal. jap.* **42**, 491.

PARNAS I. K. und WAGNER R. (1921) Über die Ausführung von Bestimmungen kleiner Stickstoffmengen nach Kjeldahl. *Biochem. Z.* **125**, 253.

PÄTAU K. (1943) Zur statistischen Beurteilung von Messreihen. *Biol. Zbl.* **63**, 152.

PIRIE A. und VAN HEYNINGEN R. (1956) *Biochemistry of the Eye.* Ryerson Press, Toronto.

SCHNEIDER W. C. (1945) Phosphorus compounds in animal tissues—I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

SCHWINCK I. (1956) Vergleich des Redox-Pigmentes aus Chromatophoren und Retina von Sepia officinalis mit Insektenpigmenten der Ommochromgruppe. *Verh. dtsch. zool. Ges.* **49**, 71 (1955).

SEKOGUTI Y. (1953) On the fractionation of phosphorus compounds in the frog-retina. *Seiro-Seitai* **5**, 78.

SÜLLMANN H. (1956) *Auge und Tränen* in: *Physiologische Chemie*, Bd. II/2.Teil, p. 864 ff., herausgeg. von FLASCHENTRÄGER B. und LEHNARTZ E. Springer-Verlag, Berlin.

TAKAMATSU T. (1934) Photochemische Studien an der Netzhaut—II. Über die Phosphorsäurebildung und die Phosphatidveränderungen der Netzhaut bei Belichtung. *Acta Soc. ophthal. jap.* **38**, 1035.

TAKANO M. (1935) Über die Gewebsatmung der Netzhaut der Ratte—II. Über die Einflüsse der kurzen Beleuchtung des farblosen Lichtes und der dauernden Beleuchtung des farbigen Lichtes auf die Gewebsatmung und aerobe Glykolyse der Netzhaut. *Acta Soc. ophthal. jap.* **39**, 162.

TAWARA M. (1938) The effect of light upon the phosphoric acid fractions of the retina. *Acta Soc. ophthal. jap.* **42**, 106.

WALD G. (1953) The biochemistry of vision. *Annu. Rev. Biochem.* **22**, 497.

WALD G. und HUBBARD R. (1957) Visual pigment of a decapod crustacean: the lobster. *Nature, Lond.* **180**, 278.

WALTHER J. B. und DODT E. (1957) Elektrophysiologische Untersuchungen über die UV-Empfindlichkeit des Insektenauges. *Experientia* **13**, 333.

WALTHER J. B. und DODT E. (1959) Die Spektralsensivität von Insekten-Komplexaugen im Ultraviolett bis 290 mμ. *Z. Naturf.* **14b**, 273.

VOL. 4 1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 304 *to* 318. *Pergamon Press Ltd., London. Printed in Great Britain*

DIAPAUSE IN THE EUROPEAN CORN BORER, *PYRAUSTA NUBILALIS* (HÜBN.)*

S. D. BECK and W. HANEC

University of Wisconsin

(*Received* 11 *December* 1959)

Abstract—Diapause in mature larvae of the European corn borer was found to be associated with three readily discernible characteristics: (1) arrested gonadal development; (2) failure to pupate shortly after cessation of larval feeding; (3) reduction of oxygen consumption to about 25 per cent that of non-diapause mature borer larvae. Diapause was found to be induced by photoperiods of from 9 to 15·5 hr of light per 24-hr period. Within this range of photoperiods, incidence of diapause was inversely proportional to the rearing temperature employed, except that with 10·5–13·5 hr photoperiods all larvae entered diapause without regard to the ambient temperature. At moderate rearing temperatures (23–25°C), the mean threshold photoperiod was 15·4 hr of light per 24 hr. The diapause threshold for photoperiods under 13 hr was not determined. The physiological changes associated with diapause were found to be largely reversible up to at least the early part of the fifth larval instar. No critical growth stage for diapause determination was detected.

INTRODUCTION

PHENOLOGICAL synchronization of the feeding larval stages of the European corn borer with the presence of suitable host plants is implemented largely by the intervention of larval diapause in the insect's life cycle.

The borer moths emerge during late spring and lay eggs on corn as the plants approach the tasselling growth stage. The young larvae feed on the leaves, tassel, and stem, becoming full grown in midsummer. Some of the borers then complete their development, emerge as moths, and lay eggs that give rise to a second generation of borers. Others, however, do not complete their development; instead, they go into a state of diapause. They remain in diapause until the following spring, at which time they pupate, emerge as moths, and start the cycle again. Of the midsummer, or second generation, all that are full grown by the end of the growing season go into diapause. Thus the overwintering population is made up of mature larvae from both generations. In north central Wisconsin the overwintering population is almost entirely of the first generation, as virtually no midsummer generation occurs. In more southern areas, such as Illinois and Indiana, the overwintering borers are predominantly from the second generation, as few of the early generation hibernate. Southern Wisconsin is in a transitional zone between the one-generation area to the north and the two-generation to the south.

* Approved for publication by the Director of the Wisconsin Agricultural Experiment Station.

As early as 1925 (BARBER, 1925) it was known that borer populations in the region of Lake Erie had but a single generation per year, whereas in the New England area two annual generations regularly occurred. This difference in seasonal life cycles was attributed to the existence of two distinct geographical races: a univoltine strain near Lake Erie and a multivoltine strain in New England. However, BARBER (1925) found that in the Lake Erie population, the percentage of individuals manifesting multivoltinism varied from zero up to 40 per cent during a 5-year study period, indicating climatic influence on the expression of voltinism.

O'KANE and LOWRY (1927) worked with the borer in New Hampshire, where two generations occur. They believed that the population was homozygous for multivoltine genetic factors, but that only a part of the population actually went through two generations. Late hatching eggs, e.g. end of July, produced larvae that entered diapause; earlier larvae developed through two generations. The authors concluded that environmental factors were apparently involved in the induction of diapause, at least in the multiple generation strain.

ARBUTHNOT (1944) found that in Connecticut the borer population was homozygous for multiple generations, while in Ohio it was mixed, containing factors for both single and multiple generations. He was able to isolate a homozygous single-generation strain from the Ohio population and found that the genetic factors responsible for the single generation were recessive. Larvae of the single-generation strain also entered the pupal stage earlier. Each strain persisted in this characteristic and multiplied to the greatest extent in climatic zones most favourable to its particular voltinism (ARBUTHNOT, 1949). KOZHANTCHIKOV (1938) could find no evidence of hereditary differences in the developmental behaviour in the Russian stocks of the borer and concluded that diapause is apparently induced by temperature conditions prevailing during larval development.

There have been a number of reports presenting evidence of change in voltinism in a given area (BRADLEY, 1947; VANCE, 1947; WRESSEL, 1953; MILLER, 1957), with supposedly univoltine populations showing an increasing multivoltinism. It has not been at all clear whether such a change has been the result of genetic shift in the borer populations or of long-term climatic cycle changes. It is certain that in many parts of the corn belt the number of generations per annum is dependent on the weather conditions during the growing season. Using the temperature accumulation method of APPLE (1952), CHIANG and HODSON (1959) showed that the occurrence and extent of a summer (second) generation of borers in Minnesota was dependent, at least in part, on early summer temperatures. They also postulated that photoperiod might be involved.

The problem of borer voltinism cannot be adequately solved in the absence of information pertaining to the identity of the factors involved in the induction of diapause in the borer. During recent years it has become apparent that diapause and other developmental changes are closely controlled by the temperature and photoperiod to which the insects are exposed (see reviews by LEES, 1955, 1956).

Photoperiodic effects on insect growth and developmental patterns are of fundamental physiological interest, in addition to their importance to phenomena

VOL. 4 1960

of voltinism, genetics, and phenology. A study was undertaken at the University of Wisconsin in 1955 to investigate the characteristics of diapause in the European corn borer and the influence of temperature and photoperiod on its incidence. As the phases of the study reported below were being concluded, Mutchmor and Beckel (1958, 1959), published the results of a somewhat similar study. The differences and similarities between their results and ours will be discussed in the appropriate paragraphs below. The two independent investigations show quite conclusively that diapause in the borer is facultative and its induction is controlled by both temperature and photoperiod.

MATERIALS AND METHODS

The corn borer larvae used in this study were reared by the aseptic method described by Beck and Stauffer (1950). The original method was modified in that instead of introducing single surface-sterilized eggs into rearing vials, the eggs were hatched on sterile agar slants and 1-day-old larvae were transferred aseptically to individual diets in shell vials (Beck, 1960).

In diapause induction studies, the larvae were reared in temperature controlled ($\pm 0{\cdot}5$°C) incubators which were equipped with lights. The lighting was provided by two 14-W daylight fluorescent lamps wired to a timer to produce any desired dark and photoperiod in a 24-hr cycle. Each vial received from 25 to about 400 ft-candles of light, depending on its position in the incubator. The variability of light intensity within the incubator did not appear to be a source of error in the experiments, as the minimum intensity (25 ft-candles) was many times greater than the apparent threshold of light response. Determinations in other species have indicated threshold values well under 5 ft-candles (Lees, 1955).

A study was initiated to determine whether a significant difference could be noted in the oxygen consumption of diapause from non-diapause larvae. The larvae for this work were collected from the field or were reared in the laboratory under controlled temperature and photoperiod. Standard manometric procedures were used (Umbreit *et al.*, 1949). Within each Warburg flask the borer larva was contained in a small wire cage. This prevented it from crawling into the centre well and also tended to keep the insect relatively quiescent, as borer larvae are strongly thigmotactic. The oxygen consumption of each larva was measured for 60 min and recorded every 10 min.

For moisture content determinations the larvae were dried to a constant weight at 90–100°C (about 48 hr). Ether extractable fat was removed by extracting the larvae with diethyl ether in a Soxhlet extractor for 48 hr.

RESULTS AND DISCUSSION

Some physiological characteristics of diapause

In undertaking a study of the factors involved in the induction of diapause, the first problem encountered was that of identifying larvae in diapause. Diapause

may be defined as a state of arrested development in which the arrested state is enforced by a physiological mechanism rather than adverse environmental conditions. Since diapause in the borer occurs in the last larval instar, the simplest criterion of diapause is a failure to pupate within a reasonable, but necessarily arbitrary, period of time after the mature larva has ceased to feed. Such a method has been used by other workers dealing with Lepidoptera with a larval diapause (DICKSON, 1949; MUTCHMOR and BECKEL, 1958, 1959; BABCOCK, 1927; FUKAYA, 1950). It has also been the principal criterion used in the present study. This method has proved to be adequate under relatively high temperature conditions ($>22°C$), as mature non-diapause larvae pupated within a few days. But at lower rearing temperatures it was found that populations of larvae presumably not in diapause required many weeks to complete pupation. This had the effect of making such experiments both less precise and more time-consuming.

An attempt was made to find other characteristics of diapause which might be used to supplement or at least verify the adequacy of the non-pupation criterion of the incidence of diapause. Larval gonadal development, oxygen consumption, fat content, and water content were studied in both non-diapause and diapause populations of borer larvae.

Gonadal development. PARKER and THOMPSON (1927) described a method for distinguishing borers in diapause from those not in diapause on the basis of gonadal development in the male larvae. Normal male larvae had large rapidly maturing testes during the fifth stadium. Larvae entering a state of diapause, on the other hand, were found to possess undeveloped rudimentary testes even after they had reached the final (fifth) larval instar and had completed the period of larval feeding and growth. The results of PARKER and THOMPSON were confirmed in the present study. Diapausing male larvae, both field collected and laboratory reared, showed greatly retarded gonadal growth. Gonadal development was not resumed until just prior to the breaking of dormancy. Although the development of the testes may be arrested in the fourth instar, the larvae could not be considered to be in diapause, since they were actively feeding and would undergo a moult to the fifth instar. The arrest of gonadal development was the earliest characteristic found which was indicative of an ensuing state of diapause. The environmental factors inducing diapause must, therefore, exert an effect at least as early as the fourth instar. This point will be discussed in more detail in a subsequent section.

Metabolism. SCHNEIDERMAN and WILLIAMS (1953, 1954) found that respiratory metabolism of cecropia pupae was strongly suppressed during diapause. Oxygen consumption of pupae in diapause was only 1·4 per cent that of the mature larvae. Such pupae were found to be relatively insensitive to carbon monoxide and cyanide poisoning. The cytochrome oxidase system appeared to be almost completely absent. Oxygen consumption increased markedly as diapause was terminated.

Oxygen consumption measurements were made on borer larvae from populations growing under different environmental conditions. The following groups

VOL. 4 1960

of larvae were studied: (a) larvae laboratory-reared at 30°C in continuous darkness (non-diapause); (b) larvae laboratory-reared under diapause inducing temperature and photoperiod; and (c) larvae collected from the field during summer, fall, and winter.

The first collections of larvae from the field were made during October, November, and December of 1955. During the summer of 1956, larvae were collected periodically from the field and their oxygen consumption and water and fat contents were determined. The field borers entered diapause after the first generation and the results indicated the metabolic pattern of a population of larvae entering diapause under field conditions. The instars of the collected larvae were determined by measuring head capsules and plotting frequency histograms.

As shown in Table 1, there was a high initial metabolic rate, high water content, and comparatively low fat content in the larvae during the earlier part of the summer. As the season progressed the metabolic rate decreased, as did the water content; however, there was an increase in the fat. Comparable data are

Table 1—Oxygen consumption, water content, and fat content of European corn borers collected during winter of 1955 and summer and fall of 1956

Collection date	No. of larvae	Instar	Oxygen consumption (mm^3/mg dry/hr)	Per cent water	Per cent fat (Dry weight basis)
24 July 1956	64	4 and 5	2·95 ± 0·85	81·7	39·3
30 July	45	5	2·34 ± 0·58	74·5	45·3
7 August	17	5	1·47 ± 0·41	66·5	52·2
14 August	42	5	1·18 ± 0·44	62·3	53·8
4 September	30	5	1·21 ± 0·45	60·3	50·2
October–December 1955	237	5	0·59 ± 0·20	52·2	—
Reared at 30°C dark (no diapause)	12	5	2·20 ± 0·38	76·6	39·0

given for mature non-diapause larvae at 30°C in darkness. The oxygen consumption, water, and fat content are comparable to those larvae collected from the field during July. The non-diapause incubator-reared larvae consumed about three to four times as much oxygen as did the diapause winter larvae collected in 1955, but only about twice as much as the September larvae.

Experiments were set up in the laboratory to determine the oxygen consumption pattern of larvae that were reared under controlled temperature and photoperiod. Non-diapause larvae were obtained by rearing them at 30°C in darkness, and diapause larvae reared at lower temperatures and short photoperiods. The results are summarized in Table 2.

Non-diapause larvae appeared to have a metabolic pattern similar to that of field larvae in the earlier instars. The early fifth-instar incubator-reared larvae

compared very well with field larvae collected during July that were also early fifth-instar or late fourth. The late fifth-instar larvae collected in the field during August and September were in diapause and their oxygen consumption was essentially similar to the late fifth instar reared under diapause-inducing conditions.

TABLE 2—OXYGEN CONSUMPTION AND WATER CONTENT OF BORER LARVAE REARED UNDER CONTROLLED TEMPERATURE AND PHOTOPERIOD

Rearing conditions	Instar	Oxygen consumption (mm^3/mg dry/hr)	Per cent water
30° C, darkness (non-diapause)	5	3·40	81·0
	4	2·96	79·6
	early 5	2·19	76·6
	late 5	2·20	68·0
20° C, darkness (diapause)	late 5	1·22	66·6
26° C, 9-hr photoperiod (diapause)	late 5	1·05	65·4

VOL. 4 1960

It is evident that as the larvae entered diapause, both in the field and under controlled rearing conditions, there was a decline in oxygen consumption and water content. Larvae in diapause used oxygen at about one-half the rate of the non-diapausing mature larvae. The drop in metabolic rate continued in the field larvae until by October the oxygen consumption was only one-quarter that of non-diapause larvae. This drop in oxygen consumption is almost identical to that reported by MUTCHMOR and BECKEL (1959), who found that the metabolic rate dropped rapidly during an 8-day period occurring after the larvae had been in the fifth instar for about 3 weeks. The more gradual decline shown in Tables 1 and 2 probably is the result of increasing proportions of the samples of larvae having undergone the 'diapause drop' in metabolism by the time the metabolic measurements were made. The diapause larvae used for the data shown in Table 2 had not been held in the fifth instar long enough for the oxygen consumption of all individuals to have dropped to its minimum. The suppression of respiration was not as strong as found in cecropia diapause, and borer larvae in diapause continue to show a significant, although reduced, sensitivity to cyanide.

An endocrinological failure and electrical silence of the central nervous system are also associated with the suppression of respiration in diapause cecropia pupae (VAN DER KLOOT, 1954, 1958). It has not yet been feasible to investigate this in the European corn borer. The arrested development of the gonads of borer larvae in diapause may be the result of an absence of a growth and differentiation hormone, as in cecropia (WILLIAMS, 1947), or possibly the presence of a diapause hormone as in the silkworm (HASEGAWA, 1957). Since borers in diapause are not

quiescent but are sufficiently co-ordinated to crawl, orient to light and contact, rebuild hibernacula, and chew (but not ingest), it is not possible that the central nervous system is not inactive.

Characteristics of diapause. Three readily detected characteristics of diapause were found in the European corn borer. The earliest to appear was an arrested gonadal development. The next characteristic to be observed was a failure to pupate within a week or two after completion of feeding. The third characteristic was a decline in oxygen consumption, which occurred several weeks after the cessation of feeding. In subsequent work, discussed below, only failure to pupate was used to distinguish diapause from non-diapause larvae.

Effects of temperature and photoperiod on incidence of diapause

Effect of temperature. Borer larvae were reared in continuous darkness at constant temperatures ranging from 15 to 30°C. The results clearly showed that low temperatures alone did not induce diapause in the majority of the larvae. Even at a continuous low temperature of 15°C, only 23 per cent of the larvae entered diapause. At 20 and 23°C, 15 and 12 per cent, respectively, entered diapause. At higher temperatures diapause incidence was negligible.

Although continuous low rearing temperatures were relatively ineffective in inducing diapause, the possibility existed that shocks of extreme low temperature during the period of development might induce diapause. This hypothesis was tested and was found to be untenable. One group of about 100 larvae were reared at 30°C in darkness and chilled at 4°C for 24 hr every third day until pupation began; only about 5 per cent of these entered diapause. Chilling the larvae at 1°C on the same schedule resulted in higher mortality but no increase in diapause incidence. Lower chilling temperatures could not be used because mortality was almost complete during the early instars. Rearing larvae at 26°C either in darkness or under a 9-hr photoperiod and combined with a single 9-hr period of chilling the fifth-instar larvae at −5° resulted in a 13 per cent increase in the incidence of diapause in both situations.

From these experiments it was concluded that low temperatures alone were insufficient stimuli to induce diapause in the majority of the larvae. Periodic mild chilling also had very little effect, and drastic chilling during the feeding period caused high mortality.

Combined effects of temperature and photoperiod. A group of preliminary experiments was run in which borer larvae were reared at different temperatures and at photoperiods ranging from 9·0 to 15·0 hr of light per 24-hr period. It was apparent (Table 3) that the incidence of diapause at any given temperature (20–29°C) was dependent on the photoperiod employed. The incidence of diapause was largely independent of temperature when the borers were reared at photoperiods of from 10·5 to 13·5 hr of light per day. At these photoperiods, nearly all of the larvae went into diapause. At photoperiods of 9·0 and 15·0 hr of light per 24 hr, the incidence of diapause tended to be inversely proportional to the temperature employed. Mutchmor and Beckel (1958, 1959) also found very high incidence

of diapause among borers exposed to 65°F (18·3°C) at photoperiods of from 9·5 to 14 hr of light per 24 hr. Because they obtained no diapause at 75°F (23·9°C) under photoperiods of 0, 8, 16, and 24 hr, they (1959) concluded that 'evidently diapause in the borer is prevented by high temperatures, regardless of the photoperiod'. The photoperiod range in which diapause is most effectively induced appears to be greater than 8 hr and less than 16 hr. Thus, MUTCHMOR and BECKEL omitted the photoperiods of interest in their 75°F series.

TABLE 3—INCIDENCE OF DIAPAUSE IN THE EUROPEAN CORN BORER LARVAE REARED UNDER DIFFERENT TEMPERATURES AND PHOTOPERIODS

Rearing temperatures (°C)	Photoperiod		No. of larvae	Incidence of diapause	
	Light (hr)	Dark (hr)		Exposed insects (%)	Dark (control) (%)
29	9·0	15·0	124	11·3	0·0
26	9·0	15·0	100	33·2	2·8
23	9·0	15·0	83	70·9	15·0
20	9·0	15·0	156	100·0	15·0
29	10·5	13·5	55	96·4	0·0
29	12·0	12·0	125	84·0	0·0
26	12·0	12·0	86	96·5	0·0
23	12·0	12·0	68	100·0	12·3
29	13·5	10·5	46	100·0	0·0
29	15·0	9·0	66	24·2	0·0
26	15·0	9·0	98	48·2	0·0
23	15·0	9·0	88	83·8	0·0

A detailed determination was made of the relationship between temperature and photoperiod effects on the incidence of diapause. Diapause incidence was measured at temperatures from 18 to 29°C under photoperiods of from 13·5 to 16·0 hr per 24 hr. Control larvae were reared under continuous darkness. The results of this series of experiments are presented in Fig. 1.

The temperature had no influence on the incidence of diapause when the larvae were reared under a photoperiod of 13·5 hr, as all of the insects entered a state of diapause. Under longer photoperiods, diapause incidence was reduced at the higher rearing temperatures. The 16-hr photoperiod and different temperatures produced a diapause incidence almost identical to that observed in borer cultures reared in continuous darkness (0-hr photoperiod). Within the range of temperatures tested, no particular temperature was found to be critical in the sense that a small change would produce a large difference in the proportion of the borers entering diapause. In regard to photoperiods, however, a sharp decline in diapause incidence occurred between 15·0- and 15·5-hr photoperiods. At 23°C,

VOL. 4 1960

for example, 18 per cent of the borers went into diapause when under a 15·5-hr photoperiod. Under a 15·0-hr photoperiod at the same temperature, diapause incidence was 83 per cent. Mutchmor and Beckel (1959) also detected the existence of a critical photoperiod, but postulated that it lay between 14·5 and

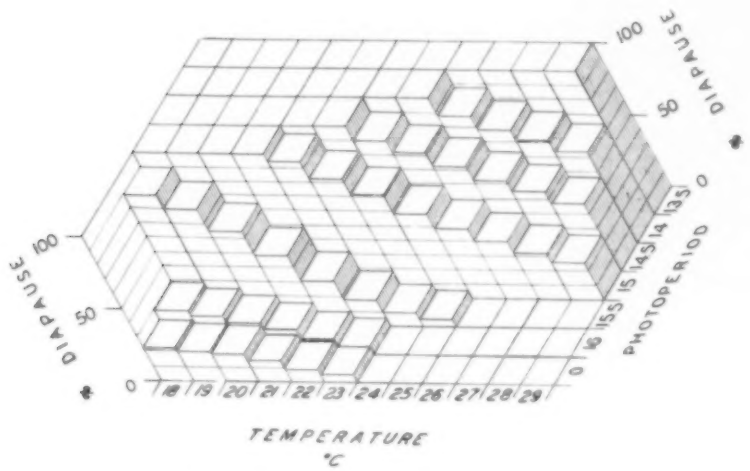


Fig. 1. Interaction of temperature and photoperiod on the incidence of diapause in the European corn borer larvae.

15·0 hr of light per 24-hr day. However, they tested neither 14·5- nor 15·0-hr photoperiods. More detailed experiments clearly show (Fig. 1) that the critical photoperiod lies between 15·0 and 15·5 hr of light per 24 hr. At temperatures of 20°C and lower, the critical photoperiod may be between 15·5 and 16·0 hr.

In the interpretation of the results discussed above, the concept of a critical photoperiod was employed in deference to established terminology. It is important that the concept be examined, as its usual use is somewhat ambiguous. The ambiguity arises from the fact that experiments on photoperiod and the incidence of diapause have been carried out on populations of insects. The occurrence of diapause of each individual insect must be a response to stimuli provided by the ambient temperature and photoperiod. The critical photoperiod would then be the response threshold of that individual. On a population basis, the critical photoperiod would be detected as the shortest photoperiod exceeding the mean response threshold of the population.

The average effect of photoperiod on diapause incidence over the temperature range 18–29°C, is shown in Fig. 2. Also shown in this figure is the average effect of temperature over a 14·0–16-hr range of photoperiods. The effect of temperature was essentially linear. The nearly linear effect of temperature on diapause incidence over the range of effective photoperiods (Fig. 2) is indicative of a simple thermodynamic influence on rate of growth. The average Q10 value for the

effect of temperature changes between 18 and 29°C was 2·65. Such a Q10 is well within the range observed for many biological and chemical processes.

The photoperiod response curve (Fig. 2) is obviously sigmoid. Using the photoperiod response data from an intermediate temperature range (23–25°C)

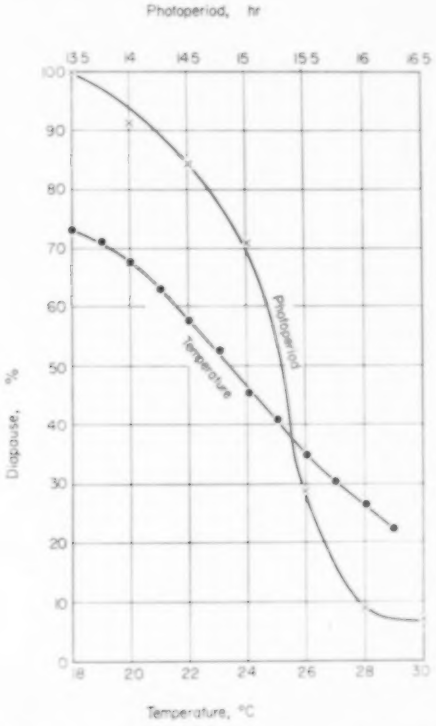


FIG. 2. Average effects of photoperiod (at temperature of 18–29°C) and temperature (at photoperiods of 14–16 hr) on the incidence of diapause among larvae of the European corn borer.

increments of per cent response approximated a normal distribution with a mean of about 15·4 hr (Fig. 3). With the experimental design employed in this study, it was not possible to obtain data from the number of photoperiods required for statistical demonstration of normally distributed responses. Although the curve may be slightly skewed toward the shorter photoperiods, a normal distribution is strongly suggested. According to this distribution, about two-thirds of the experimental population showed a diapause threshold that lay between 15·0 and 15·8 hr of light per 24 hr, at temperatures of from 23 to 25°C. The diapause thresholds were found to be temperature-sensitive (Fig. 1), with the incidence of diapause being inversely proportional to the temperature at photoperiods greater than 13·5 hr.

VOL. 4 1960

Normally distributed diapause thresholds are indicative of a borer population which is heterozygous for genetic factors controlling diapause. Arbuthnot (1944) obtained results demonstrating genetic factors in diapause manifestation in the borer. Harvey (1957) isolated by artificial selection a facultative diapause strain of spruce budworm, *Choristoneura fumiferana* (Clem.), an insect usually considered to have an obligatory diapause. The results of many other workers support the

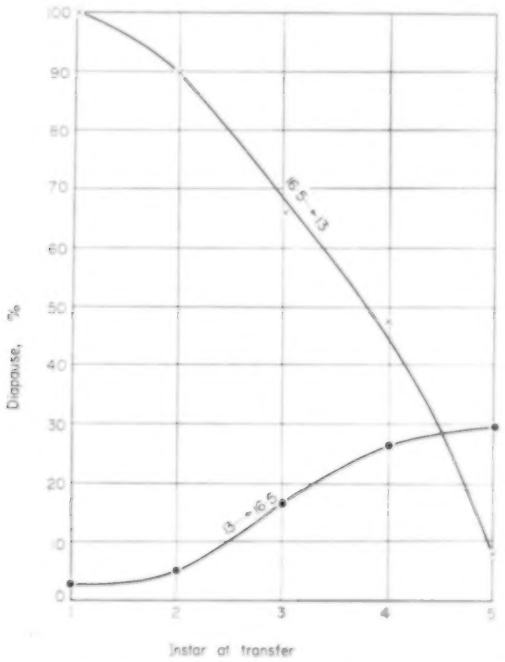


Fig. 3. Distribution of photoperiodic effects of the incidence of diapause in the European corn borer (23–25°C).

interpretation that diapause in a wide range of species is a genetically controlled physiological phenomenon (see Lees, 1955). The results of the present study are consistent with those of Arbuthnot, except that our results show that diapause in the borer is entirely facultative. In the light of the wide range of response observed (Fig. 1), the natural selection of geographical races of the European corn borer differing in mean diapause threshold is easily visualized.

Effect of growth stage on photoperiodic induction of diapause

In any consideration of the theoretical and practical implications of photoperiod on diapause incidence in the European corn borer, it is important to determine the relationships between the growth stage of the larvae and the influence at photoperiod. A series of experiments was conducted in which larvae

were reared at 26°C and under either a 16·5-hr photoperiod (non-diapause) or a 13-hr photoperiod (diapause-inducing). Systematic transfers of larvae were made from one photoperiod to another. When half of the larvae in a random sample had moulted and advanced an instar, the group was transferred and allowed to complete its development under the opposite photoperiod.

No sharply critical stage was found (Fig. 4), and all transfers made after the first instar influenced the ultimate incidence of diapause. Fifth-instar transfer from long to short photoperiod induced diapause in fewer than 10 per cent. Diapause could be prevented in up to 70 per cent of the larvae by transferring them from the short to the long photoperiod as late in development as the fifth

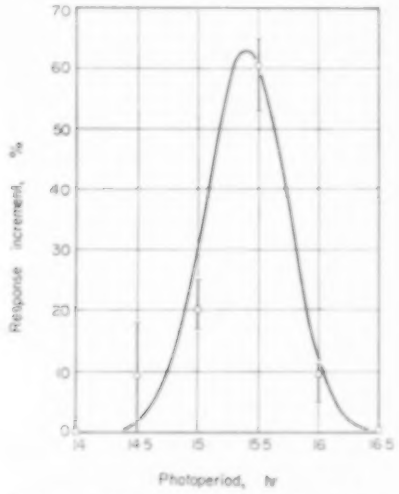


FIG. 4. Incidence of diapause among European corn borer larvae exposed to different photoperiods at different growth stages.

instar. The diapause-inducing effect of a 13-hr photoperiod appeared to be largely reversible, even as late in the life cycle as the early fifth instar. The 16-hr photoperiod promoted uninterrupted development, and this effect was not reversible in the fifth instar and only partially reversible earlier in the life cycle.

These results are in apparent disagreement with those of MUTCHMOR and BECKEL (1959), who found that diapause induction was limited to the late fourth and early fifth instars. The experimental procedure of MUTCHMOR and BECKEL was quite different from that used in the present study, in that they used: (1) continuous light as non-diapause conditions (we used a 16·5-hr photoperiod); (2) a 9·5-hr photoperiod for diapause induction (we used a 13-hr photoperiod); (3) 29°C for non-diapause and 18°C for diapause induction (we used 26°C throughout); and (4) they exposed the larvae to diapause-inducing conditions during single stadia; whereas, we exposed them to altered conditions from the point of transfer to the completion of the experiment. The disparity of results

may be explicable on the basis of procedural differences between the two studies. This is now being investigated.

Short daily exposures to long wavelength visible light were found to have a retarding effect on the growth rate of several insect species (Ball, 1958). De Wilde *et al.* (1959) obtained evidence that photoperiodic induction of diapause in the Colorado potato beetle, *Leptinotarsa decemlineata* Say, was modified in controlled feeding experiments. In view of these observations and the effects of temperature,

Table 4—Effect of photoperiod on growth rate and incidence of diapause in European corn borer reared at 26°C

Photoperiod (hr light/24 hr)	Average instar at indicated age (days) 3	7	10	14	Age at beginning of pupation (days)	Age at completion of pupation (days)	Incidence of diapause (%)
13·0	1·1	2·6	3·4	4·3	—	—	100
14·0	1·2	2·5	3·3	4·4	16	25	85
15·0	1·1	2·6	3·5	4·5	16	24	42
16·0	1·1	2·4	3·4	4·4	16	25	3

as discussed above, the effect of photoperiod on the growth rate of European corn borer larvae was investigated (Table 4). The results were conclusive; at a constant temperature, the growth rate was not modified by photoperiods ranging from 13 to 16 hr of light per 24-hr period.

Temperature, photoperiod, and voltinism

Under natural field conditions, photoperiod and temperature determine the extent to which populations of the European corn borer display univoltine and multivoltine characteristics. It is well established that the borer tends to be univoltine in the northern areas of the corn belt, and multivoltine in southern areas (Arbuthnot, 1949). That midsummer day lengths are greater in the north than in the south is also well known. On the basis of photoperiod alone, it would be expected that northern borer populations would be multivoltine. The relationship between temperature accumulation and borer development (Apple, 1952) and the modifying effect of temperature on photoperiodic induction of diapause (Figs. 1, 2) may be sufficient to account for the observed voltinism patterns. For instance, in central Iowa Civil Day Length will have decreased to 15·4 hr on 1 August. This day length is reached on 6 August in southern Wisconsin. In a normal growing season temperature accumulation at the time that day length reaches 15·4 hr is about 1750 borer degree days in central Iowa and about 1570 borer degree days in southern Wisconsin. Therefore, at comparable photoperiods borer development in central Iowa will be ahead of that in southern Wisconsin. Because day lengths become shorter as the season progresses, Iowa borers will be under longer photoperiods during their larval development than will Wisconsin borers.

The present state of knowledge concerning the sensitivity of different larval instars to diapause-inducing photoperiods and temperatures is not sufficient to enable the formulation of practical methods of predicting the occurrence and extent of multivoltinism in a particular field population during any given growing season. The problems involved in such forecasting are now under intensive study.

REFERENCES

Apple J. W. (1952) Corn borer development and control on canning corn in relation to temperature accumulation. *J. econ. Ent.* **45**, 877–879.

Arbuthnot K. D. (1944) Strains of the European corn borer in the United States. *Tech. Bull. U.S. Dep. Agric.* **869**.

Arbuthnot K. D. (1949) Temperature and precipitation in relation to the number of generations of European corn borer in the U.S. *Tech. Bull. U.S. Dep. Agric.* **987**.

Babcock K. W. (1927) The European corn borer, *Pyrausta nubilalis* (Hübn.)—I. A discussion of its dormant period. *Ecology* **8**, 45–59.

Ball H. J. (1958) The effect of visible spectrum irradiation on growth and development in several species of insects. *J. econ. Ent.* **51**, 573–578.

Barber G. W. (1925) Remarks on the number of generations of the European corn borer in America. *J. econ. Ent.* **18**, 496–502.

Beck S. D. (1960) The European corn borer, *Pyrausta nubilalis* (Hübn.), and its principal host plant—VII. Larval feeding behavior and host plant resistance. *Ann. ent. Soc. Amer.* **53**, 206–212.

Beck S. D. and Stauffer J. F. (1950) An aseptic method for rearing European corn borer larvae. *J. econ. Ent.* **43**, 4–6.

Bradley W. G. (1947) The change from the single-generation to the two-generation behavior in the European corn borer. *Proc. No. Centr. Br. Amer. Assoc. econ. Ent.* **2**, 25–27.

Chiang H. C. and Hodson A. C. (1959) Summer pupation of the European corn borer and the seasonal temperature. *J. econ. Ent.* **52**, 409–411.

Dickson R. C. (1949) Factors governing the induction of diapause in the Oriental fruit moth. *Ann. ent. Soc. Amer.* **42**, 511–537.

Fukaya H. (1950) On the factor inducing the dormancy of the rice borer, *Chilo simplex* Butler. *Proc. 8th Int. Congr. Ent.* **1**, 223–225.

Harvey G. T. (1957) The occurrence and nature of diapause-free development in the spruce budworm, *Choristoneura fumiferana* (Clem.). *Canad. J. Zool.* **35**, 549–572.

Hasegawa K. (1957) The diapause hormone of the silkworm, *Bombyx mori*. *Nature, Lond.* **179**, 1300–1301.

Kozhanchikov I. V. (1938) Geographical distribution and physiological characters of *Pyrausta nubilalis* Hb. *Zool. Zh.* **17**, 246–259.

Lees A. D. (1955) *The Physiology of Diapause in Arthropods*. Cambridge University Press.

Lees A. D. (1956) The physiology and biochemistry of diapause. *Annu. Rev. Ent.* **1**, 1–17.

Miller L. A. (1957) Changes in insect populations in Southwestern Ontario. *Rep. ent. Soc. Ontario* **87**, 15–19.

Mutchmor J. A. and Beckel W. E. (1958) Importance of photoperiod and temperature in inducing diapause in the European corn borer, *Pyrausta nubilalis* (Hübn.). *Nature, Lond.* **181**, 204.

Mutchmor J. A. and Beckel W. E. (1959) Some factors affecting diapause in the European corn borer, *Ostrinia nubilalis* (Hübn.). *Canad. J. Zool.* **37**, 161–168.

O'Kane W. C. and Lowry P. R. (1927) The European corn borer; life history in New Hampshire 1923–1926. *N. H. Agric. Exp. Sta. Tech. Bull.* **33**.

VOL. 4 1960

Parker H. L. and Thompson W. R. (1927) A contribution to the study of hibernation in the larva of the European corn borer (*Pyrausta nubilalis* Hb.). *Ann. ent. Soc. Amer.* **21**, 10–20.

Schneiderman H. A. and Williams C. M. (1953) The physiology of insect diapause—VII. The respiratory metabolism of the cecropia silkworm during diapause and development. *Biol. Bull., Woods Hole* **105**, 320–344.

Schneiderman H. A. and Williams C. M. (1954) The physiology of insect diapause—IX. The cytochrome oxidase system in relation to the diapause and development of the cecropia silkworm. *Biol. Bull., Woods Hole* **106**, 238–252.

Umbriet W. W., Burris R. H., and Stauffer J. F. (1949) *Manometric Technique and Tissue Metabolism*. Burgess, Minneapolis.

Vance A. M. (1947) What effect has the change from single-generation to two-generation behavior had on borer accumulation in dent corn? *Proc. No. Centr. Br. Amer. Assoc. econ. Ent.* **2**, 27–28.

van der Kloot W. G. (1955) The control of neurosecretion and diapause by physiological changes in the brain of the cecropia silkworm. *Biol. Bull., Woods Hole* **109**, 276–294.

van der Kloot W. G. (1958) The resting potentials of neurons in the cecropia brain during diapause and development. *Proc. 10th Int. Congr. Ent.* **2**, 79.

Wilde J. de, Duintjer C. S., and Mook L. (1959) Physiology of diapause in the adult Colorado potato beetle (*Leptinotarsa decemlineata* Say)—I. The photoperiod as a controlling factor. *J. Ins. Physiol.* **3**, 75–85.

Williams C. M. (1947) The cytochrome system in relation to diapause and development in the cecropia silkworm. *Anat. Rec.* **99**, 591.

Wressell H. B. (1953) Increases of the multivoltine strain of the European corn borer, *Pyrausta nubilalis* (Hbn.) (Lepidoptera: Pyralidae), in Southwestern Ontario. *Rep. ent. Soc. Ontario* **83**, 43–47.

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 319 *to* 347. *Pergamon Press Ltd., London. Printed in Great Britain*

THE NUTRITIONAL REQUIREMENTS OF LOCUSTS—I DEVELOPMENT OF SYNTHETIC DIETS AND LIPID REQUIREMENTS

R. H. DADD

Department of Zoology and Applied Entomology,
Imperial College of Science and Technology

(*Received* 12 *December* 1959)

Abstract—Hatchlings of *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. may be reared to the adult stage on a diet consisting of cellulose, sucrose, dextrin, a salt mixture, cholesterol, linoleic acid, casein, peptone, egg albumen, ascorbic acid, and ten water soluble vitamins of the B complex. In addition β-carotene is required for pigmentation in *Schistocerca*. In developing this diet ascorbic acid was shown to be essential for *Schistocerca*, and it was found necessary to provide a mixture of proteins as casein alone was inadequate for good growth. A crucial factor in ensuring consistently good growth was the presence of high amounts of indigestible material. This was provided as cellulose in amounts sufficient to give the diet utilizability of the order found for grass. A combination of cholesterol and linoleic acid satisfied the lipid requirements of both species for growth to the adult stage. Tocopherol (vitamin E) was not required, and although pigmentation in *Schistocerca* depended on dietary carotene, this substance was without effect on larval growth. In general, *Locusta* was more sensitive to lipid deficiencies than was *Schistocerca*.

VOL. 4 1960

INTRODUCTION

In recent years the development of synthetic diets for several insect species has afforded considerable insight into the nutritional requirements of the class. From this information, surveyed in several reviews (Trager, 1953; Hinton, 1956; Lipke and Fraenkel, 1956; Levinson, 1955), it seems that insects are qualitatively very uniform in their nutritional requirements. Basically similar to vertebrates, they differ as a class in being unable to synthesize steroids and in requiring no vitamins other than certain of the B complex. In spite of the extreme variety of food substances used by insects, nutritional differences relate largely to the proportions of bulk components of the food, the fats, carbohydrates, proteins, water and indigestible matter, and to the relative importance of micro-organisms, external and symbiotic, in supplying vitamins.

This apparent nutritional uniformity may be partly a result of similarity in the type of insect selected for study, for if the species for which extensive information is available are grouped according to their natural foodstuff, it appears that most infest stored products or are Diptera whose larvae develop in fluid or semi-fluid environments. Information is particularly meagre on the large group of phytophagous insects; a recent review on this topic (Friend, 1958) cites only four species that have received detailed study using highly purified diets.

A major reason for the paucity of data on this important group is the difficulty of devising artificial foods that will be eaten by them. Many require specific phagostimulant factors, chemical or physical, to initiate and maintain feeding (THORSTEINSON, 1958; DE WILDE, 1958), thus presenting a prior behavioural problem. It is probably more than coincidence that the four successfully studied phytophagous species listed by FRIEND (1958) are all plant borers. The provision of a solid medium simulating physical conditions within plant tissues is comparatively simple compared with the preparation of a diet having the external properties of plants, which may, in addition to suitable texture and turgidity, require the provision of sharp edges, crevices, laminae, a dry surface, and special orientation in relation to light or gravity. It is not surprising therefore that only one leaf-eater, the Southern army worm, *Prodenia eridania*, has been amenable to nutritional studies based on artificial food (ELLIOTT, 1955).

A severe limitation on work with *Prodenia* which also applied to the plant-borers *Pyrausta* (BECK *et al.*, 1949), *Chilo* (ISHII, 1952), and *Laspeyresia* (MATSUMOTO and YASUE, 1953), arose from the need to include in the artificial diet crude plant extracts in high proportion. In supplying unknown growth factors, these plant adjuvants, if present in considerable amount, mask and prevent evaluation of vitamin and amino acid requirements. Furthermore, they may introduce growth inhibitors along with growth promoters, as was the case with *Pyrausta* (BECK, 1953, 1957).

Similar difficulties attended the earlier work on non-phytophagous insects, where yeast extracts were often necessary components of diets. In subsequent studies pure substances were sometimes found suitable as replacements for these 'unknown yeast factors'. Successive investigations into the nutrition of *Aedes* are instructive in this connexion (GOLBERG *et al.*, 1945; GOLBERG and DE MEILLON, 1948a, b; TRAGER and SUBBAROW, 1938; SUBBAROW and TRAGER, 1940; TRAGER, 1948; SINGH and BROWN, 1957). At present the factors supplied by plant preparations remain unidentified. In the case of *Pyrausta* they are not known vitamins or amino acids (BECK, 1953).

In attempting to formulate an artificial food for use with locusts it seemed unlikely that the initiation of feeding would involve very specific stimuli as these insects thrive on a wide range of food plants (HUSEIN *et al.*, 1949), avidly eat bran (DAVEY, 1954), and when hungry attack such diverse materials as lump sugar, wood, paper, and picene wax. When preliminary tests showed that hoppers of both *Schistocerca* and *Locusta* willingly ate strips of 1 per cent agar containing sugar, casein, and yeast, it seemed that a diet based on the medium devised for *Pyrausta* (BECK *et al.*, 1949) might prove a satisfactory starting-point. This paper deals with the evolution of a series of diets of increased purity and adequacy from such a formulation.

MATERIALS AND METHODS

The species of locust used, *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L., are kept in culture at the laboratories of the Anti-Locust Research Centre, London. In testing the earliest diets growth experiments were started with third instar hoppers, as there was

little point in attempting to rear from the egg until a diet which supported growth during the later larval stages had been obtained. These third instar hoppers were taken from gregarious breeding stocks where their food had been grass supplemented with bran. When hatchlings were eventually required, these were at first collected on the day of hatching and used within 24 hr. Later, egg pods were obtained a day or two before using and hatched in the author's laboratory, thereby, it was hoped, avoiding adverse effects from excessive early handling.

In growth trials starting at the third instar, hoppers were kept isolated in 1·5 litre glass jars with plastic-gauze, screw-on lids. When starting with hatchlings, gregarious conditions were usually maintained, with 15–30 hoppers per jar in the first instar, 10–15 per jar in the second and third instar, and 5–10 thereafter. Watkin and Doncaster celluloid cages of about 12 litre capacity were sometimes used, with a density of 20–50 hoppers per cage.

All jars and cages were supplied with perches. At first these were splints of boxwood, but when it became apparent that considerable amounts of wood were sometimes eaten, strips of perforated zinc were substituted. Experiments took place in a constant temperature room at $32 \pm 0{\cdot}5$°C, permanently illuminated, and with a controlled ambient relative humidity of 55 ± 5 per cent. Humidity within the jars must have been considerably higher, as all contained an open water supply in the form of moist cotton wool. It was expected that the gradient of humidity from saturation at the water pad to 55 per cent r.h. at the gauze lid would encompass the region of 60–70 per cent r.h. considered optimal for *Schistocerca* and *Locusta* (Hamilton, 1950).

Diets were prepared as follows. Weighed lipids were dissolved in ether or chloroform in a mortar. Casein, cellulose, and yeast were weighed into the solution and the whole intimately ground as the ether evaporated, this process being speeded up in an oven at 60°C. For the agar gel diets used initially, salts and sugars were weighed out and dissolved in the appropriate amount of previously prepared agar solution. As the agar cooled below 60°C water soluble accessory factors were dissolved in it, and when the temperature had dropped to about 40°C the casein–cellulose–yeast mixture was added and thoroughly stirred in. At the point of gelation the mixture was poured on to suitable sized Whatman's No. 1 filter papers in a large petri dish, so that when set a block of gelled diet interleaved with filter papers was obtained. This type of diet was dispensed by tearing off strips of filter paper with an adhering layer of gel and draping them on the perches in the rearing jars.

For the dry diets which superseded these agar preparations the casein–cellulose–yeast–lipid fraction was prepared in the same way. Other bulk components (salts, sugars, albumen, peptone, nucleic acids, etc.) were ground together to a fine powder before being mixed with the first fraction. Water soluble accessory factors made up in solution of appropriate concentration were added, and the whole ground to a friable mixture of even consistency. Dry diets were dispensed in small petri dishes placed at the bottom of the rearing jars on the cotton wool water pads. All diets were stored in a deep freeze. Vitamin mixtures were made up in stock solution in amounts sufficient for 2–3 weeks, being stored during this time in the deep freeze. Calcium pantothenate and *p*-aminobenzoic acid were dissolved in 20 per cent alcohol and the pH adjusted to 7·0; all other B vitamins were made up in 20 per cent alcohol and adjusted to about pH 3·5.

The adequacy of diets was evaluated by comparing mean growth rates and percentage survival to the adult stage of equivalent groups of hoppers reared on them. The best diet available was included as a control until a modification giving improved performances superseded it. In setting up equivalent groups of hoppers for an experiment starting at the third instar all were weighed and divided so as to give as nearly as possible similar weight distributions between the groups. When hatchlings were used, equivalent groups were made up by splitting the hoppers emerging from each egg pod equally between the groups.

The groups of hoppers thus obtained were placed in rearing jars at the appropriate density, supplied with perch, water-pad, and the requisite diet, and left in the constant

VOL. 4 1960

temperature room. When using agar-based diets, water pads and diet had to be changed daily, as strips of diets hanging on the perches dried out to a tough, inedible consistency in 6–8 hr and scraps falling on to the water pad became sour if left more than a day. With dry diets fresh food and water pads were supplied every 2 days, the pads being moistened in the intervening days. Growth data were obtained by weighing the hoppers individually and noting their stadium from time to time, usually every 2 days when the jars were cleaned out and given fresh food and water.

No attempt was made to sterilize the diets or to provide aseptic rearing conditions. This would have been superfluous in the initial work using diets containing much yeast, and when dry diets proved acceptable it was hoped that their low water content would be unfavourable for the multiplication of micro-organisms. In the event, the successful use of dry unsterilized synthetic diets to demonstrate requirements for the major B vitamins has justified this hope.

DEVELOPMENT OF SYNTHETIC DIETS

The composition of an agar-based diet which first enabled isolated third instar hoppers of *Schistocerca* to grow to adults is given in Table 1(A). On this and similar diets *Locusta* hoppers grew poorly, so attention was concentrated on *Schistocerca*. Attempts to rear hatchlings on this diet were unsuccessful, all dying by the third instar.

In these experiments the colour of third instar hoppers given artificial food came to differ strikingly from that of controls fed on grass. Whereas grass caused an intense yellow body colour to develop during the fourth and fifth instars, artificial food induced a progressive pallor with each moult. In both cases black markings tended to disappear, but this is a well-known consequence of isolation (GUNN and HUNTER-JONES, 1952). As the yellow colour of *Schistocerca* hoppers is due to carotenoid pigments supposedly derived from green plant tissue (GOODWIN, 1952), the effect of supplying β-carotene in the artificial diet was examined. As anticipated, this enabled third instar hoppers to develop normal pigmentation in the later stages and growth was only slightly inferior to that of controls fed on grass.

When an attempt was made to rear hatchlings on this carotene-augmented diet, though many grew moderately during the early instars, mortality was very heavy during the third instar and none survived the fourth. It appeared unlikely that failure was due to reduced feeding owing to unpalatability, for the growth of hoppers taken from grass at the third instar had appeared nearly normal. More probably the diet lacked some substance which could be assimilated in sufficient quantity from grass during the first two instars to support good growth thereafter.

The nature of the deficiency was eventually indicated by an experiment with various modifications of the carotene-augmented diet; one of these containing ascorbic acid and choline chloride enabled twelve out of thirty-two hatchlings to become adults, whereas with the remainder mortality was 100 per cent by the fourth instar. The individual effects of choline and ascorbic acid were subsequently examined in experiments where they were omitted singly from a control diet, the formulation of which is given in Table 1(B). This and all subsequent diets were prepared without agar as friable mixtures of low water content, as growth

was thereby unimpaired. The change to dry diets enabled food to be stored for longer periods without deterioration, and wastage in the rearing jars was reduced.

TABLE 1—THE COMPOSITION OF SOME ARTIFICIAL DIETS

Substance	Diets referred to in text				
	A	B	C	D	E
Water (ml) (20% ethanol where vitamin mixtures were used)	100	2·5	2·5	10·0	10·0
Agar (g)	0·5				
Cellulose powder (Light and Co.) (g)	2·0	2·0	2·0	5·0	15·0
Water soluble chlorophyll paste (g) (B.D.H.)	0·5				
Wheatgerm oil (Vitamins Ltd.) (ml)	0·2	1·0	1·0	1·0	1·0
Cholesterol (g)	0·2	0·5	0·5	0·2	0·2
β-carotene (mg) (Light and Co., 'natural' in diets A, B; 'synthetic' in diets C, D, E)		25	25	25	25
Salt mixture (g)*	0·5	0·5	0·5	1·0	1·5
Glucose (g)	5·0	5·0	5·0		
Sucrose (g)	5·0	5·0	5·0	15·0	5·0
White Dextrin (g) (Hopkins and Williams)					5·0
Casein, fat and vitamin free (B.D.H.) (g)	3·0	3·0	6·0	6·0	6·0
Bacteriological peptone (B.D.H.) (g)				2·0	2·0
Egg albumen powder (g) (B.D.H.)				2·0	2·0
Debittered yeast powder (Glaxo) (g)	2·0	3·0			
Ascorbic acid (mg)		100	100	100	100
Choline chloride (mg)		100	100	50	50
Thiamine (μg/g diet)			25	25	25
Riboflavin (μg/g diet)			25	25	25
Nicotinic acid (μg/g diet)			50	50	100
Pyridoxine (μg/g diet)			25	25	25
Folic acid (μg/g diet)			75	25	25
Meso-inositol (μg/g diet)			100	250	250
Ca. pantothenate (μg/g diet)			50	50	50
p-aminobenzoic acid (μg/g diet)			25	25	25
Biotin (μg/g diet)			1	1	1

* Steenbocks salt mixture No. 40 was used with the yeast-containing diets (A, B). Glaxos nutritional salts were used in all synthetic diets (C, D, E). The composition of these mixtures is:

Steenbocks Salts No. 40

Sodium chloride	233·6 parts
Mg. sulphate $7H_2O$	246·0 parts
$Na_2HPO_4.12\ H_2O$	358·0 parts
K_2HPO_4	696·0 parts
$Ca.HPO_4.2H_2O$	698·0 parts
Ca. lactate	154·0 parts
Iron citrate	59·8 parts
Pot. iodide	1·6 parts

Glaxos Salt Mixture DL.6

Sodium chloride	22·0 parts
Calcium phosphate	130·0 parts
Pot. citrate	125·0 parts
Magnesium sulphate Cry.	30·0 parts
Iron citrate	5·0 parts
Trace mixture (see below)	0·7 parts

Trace Mixture

Pot. iodide	12 parts
Sod. fluoride	10 parts
Anh. manganese sulphate	2 parts
Cuprous iodide	1 part
Anh. pot. alum	1 part
Zinc sulphate	1 part

VOL. 4 1960

The data given in Table 2 show that omission of ascorbic acid caused complete mortality by the fourth instar. In each of three experiments omitting choline chloride growth was poor compared with the control, though some adults were obtained. It seemed likely, therefore, that the yeast in the diet only partially satisfied the requirement for choline.

TABLE 2—THE EFFECT OF ASCORBIC ACID ON THE GROWTH OF *Schistocerca*

Treatment	Exp.	Mean wt (mg) and numbers of individuals surviving (in brackets) at these times after hatching			Adults obtained			% of 6-day larvae to become adult
		6 days	18 days	34 days	♂♂	♀♀	Aborts*	
Control diet	A	55 (20)	340 (5)	1103 (4)	1	2		15
	B	73 (31)	405 (20)	1220 (14)	7	6		42
	C	62 (34)	372 (22)	1226 (9)	4	3	1	21
No ascorbic acid	A	53 (22)	152 (8)	all dead	0	0		none
	B	61 (30)	189 (9)	all dead	0	0		none
	C	51 (30)	224 (16)	all dead	0	0		none

* Aborts: in this and subsequent tables, this term refers to individuals which attempted to moult, but failed to emerge.

Some comments on the role of carotene based on experiments using this diet have already been reported (DADD, 1957). Extended work which will be discussed later produced conflicting results which were difficult to interpret. This was largely because the control diet proved to vary widely in its adequacy for successive batches of hoppers. The ensuing search for a more satisfactory control eventually led to the elimination of yeast from formulations, as it became apparent that little precise nutritional information could be expected until a mainly synthetic diet was available.

Yeast was first replaced by an equal weight of casein and a mixture of those vitamins generally considered essential for insects, in amounts based on the maximum concentrations used by previous workers. This diet (Table 1(C)) failed to support growth beyond the third instar, and addition of various combinations of yeast nucleic acid, the amino acids glycine and cystine (present in low proportion in casein), and the vitamins menapthone (K), rutin (P), B_{12}, and carnitine was without effect.

These negative results suggested that yeast either supplied some unidentified nutrient or phagostimulant factor, or, while not fulfilling a specific requirement, might be of importance in contributing to the overall balance of nutrients. It could be imagined, for example, that were casein alone an ill-balanced source of amino acids, yeast protein (50 per cent by weight dried yeast) might adjust

this imbalance. Such considerations led to the use of diets containing mixed proteins, formulated so as to bring the proportions of the main components more into accord with the crude composition of grass shown by chemical analysis (Ministry of Agriculture Bulletin No. 124, 1947). The initial agar diets used had been designed with those analyses in mind, but in subsequent development the proportions had diverged radically.

The composition of the first 'mixed protein' diet, in which egg albumen and peptone were used in addition to casein, is given in Table 1(D). It supported fair growth and the proportion of hatchlings to become adult ranged from 5 to 50 per cent in different trials. This improvement was doubtless in part a consequence of the overall readjustment in formulation, but experiments in which different components of a protein–amino acid–nucleic acid complex were omitted showed that hoppers grew poorly and rarely completed development unless casein were supplemented with albumen and peptone; various amino acids and nucleic acid seemed without effect.

Although *Schistocerca* could now be reared to the adult stage on a largely synthetic diet, performances were as erratic as with yeast-containing diets. A variable proportion of individuals in each experimental group grew well to become adult, but most achieved only various degrees of partial development. Two hypotheses came to mind to account for this. It might be supposed that were the diet deficient in some factor present in variable, sometimes adequate, amounts in the egg, hoppers from such adequate eggs would survive and the rest succumb at various stages determined by the exhaustion of the ovarial store. Alternatively, supposing the diet nutritionally adequate, it might be that only a proportion of the hoppers were eating enough for good growth, a situation which could be accounted for in terms of variation in threshold of phagostimulation should the diet be sub-optimal with respect to normal stimuli for feeding behaviour.

An opportunity to examine the latter hypothesis arose when it was found that the addition of grass meal (ground dried grass) or grass protein (RAYMOND and TILLEY, 1956) to the diet, greatly improved growth and reduced variability between trials. By comparing the amounts of food consumed by hoppers given synthetic diets with or without grass adjuvants, and grass, it was hoped to determine whether those diets which produced the best growth did so by virtue of superior palatability.

The procedure used in these experiments allowed similar groups of hungry fifth instar hoppers continuous access to food and water for a period of 3 days, during which the daily increments in body weight, daily faecal production, and the total food taken were recorded. Faecal measurement, an index of food consumption used in studies with *Plusia* (THORSTEINSON, 1953), was expected to provide a check on the direct measurement of food consumed. The increase in weight of hoppers provided a further check on food consumption; were this increment low on a diet showing high food consumption and faecal production, a nutritional deficiency would have been indicated, but such a result was unlikely to become manifest during the limited period of the experiments.

Some data from experiments comparing grass, the synthetic diet given in Table 1(D), and a modification of this diet in which 12 g of grass protein were used to replace 2 g casein, 2 g peptone, and 2 g albumen are given in Table 3. Average weight increments after 24 hr were similar, and after 3 days were still of the same order, so it may be assumed that not very different amounts of *nutrient* material were ingested, although the dry weight of grass taken was considerably

TABLE 3—FOOD EATEN, FAECES PRODUCED AND WEIGHT INCREMENTS FOR *Schistocerca* FIFTH INSTAR HOPPERS GIVEN VARIOUS DIETS FOR 3-DAY PERIODS. EACH VALUE IS THE MEAN FOR A GROUP OF FIVE HOPPERS

	Exp.	Grass	Synthetic diet	Synthetic diet with crude grass protein
Mean wt. increase in hoppers after 24 hr (mg)	A	229, 302	257, 115	324, 469
	B	270, 235	314, 261	356, 271
Mean wt. increase in hoppers after 3 days (mg)	A	447, 680	623, 233	433, 650
	B	446, 659	430, 506	626, 606
Dry wt. of faeces per hopper during 3 days (mg)	A	436, 610	96, 51	125, 186
	B	359, 530	70, 80	199, 213
Dry wt. of food eaten per hopper during 3 days (mg)	A	684, 1068	324, 210	422, 566
	B	499, 921	327, 331	542, 638
Coefficients of digestibility*	A	36%, 43%	70%, 76%	70%, 67%
	B	28%, 42%	79%, 76%	63%, 67%

* The coefficient of digestibility is defined as:

$$\frac{\text{(Dry wt. of food eaten)} - \text{(Dry wt. of faeces produced)}}{\text{Dry wt. of food eaten}}$$

greater than that of either synthetic diet. The most marked differences occur in the amounts of faeces produced. These were very much greater on grass than on the grass-augmented synthetic diet, this being in turn greater than on the synthetic diet. These data suggested that relatively little of the synthetic diets were being passed from the gut as faeces, and on calculating digestibility coefficients it appeared that whereas about 40 per cent of grass (dry weight) was utilized, synthetic diets were utilized to the extent of 65 and 75 per cent, the lower percentage occurring where grass protein was incorporated.

These experiments failed to distinguish marked differences in palatability, but quite clearly the synthetic diets had been grossly deficient in indigestible bulk material, and it was concluded that the improved performances obtained on the diets containing grass preparations were in part due to some amelioration of this defect. It could be imagined that were roughage inadequate a constipated condition could develop with diminished intake of food resulting in poor growth, death ensuing should complete obstruction arise.

A synthetic diet of high cellulose content (Table 1(E)) was therefore formulated and performances on it were compared with the low cellulose diet (Table 1(D)) and grass, in three experiments whose results are summarized in Table 4. A consistent improvement in growth rate and in the percentage of adults obtained

TABLE 4—GROWTH DATA FOR *Schistocerca* GIVEN HIGH AND LOW CELLULOSE SYNTHETIC DIETS AND GRASS

Treatment	Exp.	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of hoppers at 6 days to become adult
		6 days	18 days	26 days	♂♂	♀♀	Aborts	
Low cellulose synthetic diet (Table 1D)	A	63 (27)	349 (16)	856 (9)	3	3		22
	B	52 (20)	466 (4)	1322 (2)	2	0		10
	C	53 (13)	340 (3)	983 (2)	0	0	1	None
Low cellulose diet with grass protein	A	77 (20)	540 (12)	1176 (8)	4	2		30
	B	85 (24)	696 (12)	1518 (10)	7	2		38
	C	79 (16)	622 (10)	1448 (8)	7	1		50
Grass only	A	75 (17)	646 (14)	1406 (13)	9	2		65
	B	111 (23)	698 (14)	1554 (9)	4	2		26
	C	99 (20)	608 (13)	1689 (6)	3	3		30
High cellulose synthetic diet (Table 1E)	A	73 (30)	571 (23)	1425 (12)	7	2		30
	B	75 (30)	622 (15)	1393 (14)	6	6		40
	C	77 (12)	542 (6)	1302 (6)	5	1		50

from the high cellulose synthetic diet is evident. Digestibility coefficients of 45–50 per cent were obtained with this diet, supporting the idea that mechanical factors concerned in alimentation could be of crucial importance in extended growth.

Subsequent work with this high cellulose diet gave consistent results with successive batches of *Schistocerca*. Moreover, *Locusta*, which had hitherto proved intractable, was able to complete development with good adult survival, though at a considerably reduced growth rate compared with a grass diet. The diet was therefore adopted as a suitable control for the detailed examination of the nutritional requirements of both species. In this paper lipid requirements are dealt with. Requirements for other classes of nutrients will be dealt with in separate publications.

Lipid requirements

1. *Carotene*. A possible involvement of carotene in the growth of *Schistocerca* was suspected when in the initial experiments with third instar hoppers the inferior growth of those given artificial diets was accompanied by a failure in

VOL. 4 1960

pigmentation. The yellow pigment concerned is β-carotene (GOODWIN, 1952), well known as a precursor of vitamin A in vertebrate nutrition. It was soon shown that the inclusion of β-carotene in the artificial diet enabled third instar *Schistocerca* to develop normal pigmentation in later instars, and data for the experiment in question indicated that growth was greatly improved (Table 5).

TABLE 5—GROWTH DATA FOR GROUPS OF EIGHT *Schistocerca* HOPPERS TAKEN FROM GRASS DIET AT THE THIRD INSTAR AND REARED THEREAFTER IN ISOLATION ON AGAR-BASED ARTIFICIAL DIETS WITH AND WITHOUT CAROTENE

Diet	Mean weights (mg) of groups of hoppers and numbers alive (in brackets)				
	On removal from stock	After isolation with artificial diets			
		6 days	12 days	18 days	24 days
Without carotene	224 (8)	517 (8)	892 (8)	1215 (8)	1153 (7)
With carotene	216 (8)	571 (8)	1027 (8)	1481 (8)	1663 (5)
Grass control	208 (8)	568 (8)	1191 (7)	1445 (7)	1716 (6)

TABLE 6—GROWTH DATA FOR *Schistocerca* HOPPERS FROM EXPERIMENTS IN WHICH CAROTENE WAS OMITTED FROM AN ARTIFICIAL DIET OR REPLACED BY VITAMIN A ACETATE OR LYCOPENE

For diet used see Table 1B; for comments see text

Exp. No.	Mean wt. (mg) of hoppers and numbers surviving 34 days after hatching				% of hoppers at 6 days to become adult			
	Carotene	No carotene	Vit. A acetate	Lycopene	Carotene	No carotene	Vit. A acetate	Lycopene
1	1077 (4)	All dead	—	—	36	0	—	—
2*	737 (2)	399 (1)	—	—	0	0	—	—
3	1227 (14)	491 (1)	—	—	42	0	—	—
4*	1093 (12)	637 (2)	—	—	27	0	—	—
5	1275 (13)	678 (3)	1392 (7)	—	35	0	16	—
6*	1226 (9)	All dead	971 (5)	—	27	0	8	—
7	1108 (11)	708 (1)	792 (10)	—	31	4	29	—
8	1276 (4)	All dead	1023 (7)	—	14	0	26	—
9*	1098 (3)	893 (7)	1117 (11)	—	10	13	37	—
10*	1462 (1)	All dead	1249 (3)	—	5	0	21	—
11	1308 (3)	1013 (6)	All dead	973 (7)	18	15	0	21
12	1160 (8)	1154 (3)	1154 (11)	1294 (7)	28	7	20	19
13	611 (6)	707 (5)	600 (2)	393 (5)	3	0	3	0
14	1156 (10)	755 (5)	690 (7)	607 (7)	27	7	7	0
15	762 (8)	551 (8)	727 (8)	664 (10)	10	10	10	17

* Ether-extracted yeast used in these diets.

When a diet which enabled hatchlings to develop to adults became available (Table 1(B)) the effect of omitting carotene was examined in a series of experiments.

Pigmentation was affected in two ways. A pink tinge which occurs in second and third instar hoppers and in newly fledged adults failed to appear unless carotene was in the diet; also, as might be expected, the yellow colour caused by carotene was absent in the fourth and fifth instars. According to GOODWIN (1952), the pink tinge of *Schistocerca* third instar hoppers and fledgelings is caused by free, reduced insectorubin. It was shown by boiling, a treatment which liberates protein-bound insectorubin and makes it visible as a diffuse pink coloration, that the substance was still present in the tissues of hoppers reared on carotene-free diets; it appears then that carotene is in some way connected with the presence of insectorubin in the free state.

Growth data for this series of experiments are summarized in Table 6. These first led to the conclusion that carotene was essential for good growth and adult development, for in experiments 1–8 the diet containing β-carotene consistently gave better growth, and only one adult was obtained from groups fed on the carotene-free diet. Initial experiments using vitamin A acetate in place of carotene (Exp. 5–8) further suggested that this substance, while being ineffective in pigmentation, could replace carotene in supporting good growth and development. However, on extending the work to check the initial vitamin A results and to determine whether lycopene (a non-pro-vitamin A carotenoid) affected growth or colour, contradictory results were obtained. It became apparent that adult development could occur without carotene or vitamin A (Exps. 7, 9, 11, 12, 14, 15). Furthermore, the complete failure of several trials in which all carotene-containing controls died as larvae showed that a final decision on the role of carotene would have to await the development of a more reliable control diet.

When the synthetic diet given in Table 1(E) was found to support consistently good growth it was used in determining the affect of various dosages of carotene. Some results of experiments using *Schistocerca* are given in Table 7, and similar data for *Locusta* are summarized in Table 8. It is clear that carotene was not required for development to the adult stage by either species nor was the growth rate affected by the range of concentrations used.

These experiments confirmed the earlier results obtained on pigmentation with *Schistocerca*. The lowest concentration of carotene had a discernible, though weak, effect on both pink and yellow colour systems. It was noticed, however, that even with carotene in high concentration the yellow colour of the fourth and fifth instars was generally less intense than had been the case using yeast-containing diets.

It was concluded from these experiments that carotene could be omitted from the basic diet in subsequent investigations unless there was reason to suppose that pigmentation effects might be involved.

2. *Fatty acids and vitamin E.* A requirement for dietary unsaturated fatty acids was demonstrated for the lepidopterous larva *Ephestia* by FRAENKEL and

VOL. 4 1960

TABLE 7—THE MEAN WEIGHTS OF GROUPS OF *Schistocerca* HOPPERS 28 DAYS AFTER HATCHING AND THE PERCENTAGE TO BECOME ADULT WITH VARIOUS CONCENTRATIONS OF β-CAROTENE IN THE DIET

Exp. No.	Mean wt. (mg) and numbers of hoppers alive (in brackets) 28 days after hatching				% of hoppers at 6 days to become adult			
	No carotene	0·03 mg/g (× 1)	0·14 mg/g (× 5)	1·4 mg/g (× 50)	No carotene	0·03 mg/g (× 1)	0·14 mg/g (× 5)	1·4 mg/g (× 50)
1	1614 (11)	1464 (8)	1374 (12)	1465 (10)	47	32	73	40
2	1490 (8)	1288 (3)	1261 (4)	1282 (6)	66	18	18	30
3	1409 (3)	1457 (2)	1442 (3)	1602 (3)	18	50	75	50
4	1557 (10)	1428 (10)	1451 (10)	1477 (16)	66	50	45	42
5	1388 (11)	1357 (12)	1317 (12)	1450 (10)	55	71	41	45
6	1253 (8)	1131 (4)	1015 (6)	1262 (2)	39	25	28	5
7	1326 (14)	1364 (3)	1406 (6)	1516 (5)	30	33	71	50

TABLE 8—MEAN WEIGHTS OF GROUPS OF *Locusta* HOPPERS 28 DAYS AFTER HATCHING AND THE PERCENTAGE TO BECOME ADULT ON DIETS CONTAINING VARIOUS CONCENTRATIONS OF β-CAROTENE

Exp. No.	Mean wt. (mg) of hoppers and numbers alive (in brackets) 28 days after hatching				% of hoppers at 6 days to become adult			
	No carotene	0·03 mg/g diet (× 1)	0·14 mg/g diet (× 5)	1·4 mg/g diet (× 50)	No carotene	0·03 mg/g (× 1)	0·14 mg/g (× 5)	1·4 mg/g (× 50)
1	1134 (12)	1015 (12)	1068 (15)	991 (16)	42	60	65	75
2	873 (8)	867 (8)	881 (14)	1056 (7)	30	35	55	35
3	944 (3)	844 (6)	794 (9)	854 (9)	27	55	48	58

BLEWETT (1946). At first this requirement was satisfied by the inclusion of wheatgerm oil. Later it was shown that linoleic, linolenic, or arachidonic acids could replace oil, and that in addition α-tocopherol, which occurs in wheatgerm oil, had a growth-enhancing function (FRAENKEL and BLEWETT, 1946). Vegetable oils were included in diets for *Pyrausta* (BOTTGER, 1942; BECK *et al.*, 1949) and *Pectinophora* (BECKMAN *et al.*, 1953; VANDERZANT and RIESER, 1956); linoleic acid containing 1 per cent α-tocopherol was subsequently substituted in the *Pyrausta* diet (BECK, 1950), while linoleic acid alone replaced cotton-seed oil for *Pectinophora* (VANDERZANT, 1957).

Wheatgerm oil was included in the initial yeast-containing diets used with *Schistocerca* to cover possible requirements for both unsaturated fatty acids and tocopherol. It was not possible to show a clear-cut need for oil in yeast diets, for though its omission caused a high proportion of abortions or deformity at the larval–adult moult, growth during the early instars was in fact improved. When a synthetic diet was available for both *Schistocerca* and *Locusta* the effect of different amounts of wheatgerm oil was examined. The value of working with two species became apparent during these experiments, as *Locusta* showed greater sensitivity to lipid deficiencies than *Schistocerca*.

VOL. 4 1960

TABLE 9—GROWTH DATA FOR GROUPS OF *Locusta* HOPPERS GIVEN VARIOUS CONCENTRATIONS OF WHEATGERM OIL

Treatment	Exp.	Mean wt. (mg) and numbers of hoppers alive (in brackets) at these times after hatching			Adults obtained			% of hoppers at 6 days to become adult
		6 days	20 days	30 days	♂♂	♀♀	Aborts	
No oil	A	54 (32)	166 (16)	336 (8)	0	0	1	None
	B	59 (27)	183 (16)	376 (10)	0	1	1	4
	C	50 (37)	183 (15)	346 (11)	1	0	1	3
5 mg oil/g diet	C	59 (26)	443 (23)	1043 (15)	10	8	1	69
25 mg oil/g diet	A	64 (27)	345 (18)	941 (13)	6	5	1	41
	B	67 (20)	327 (10)	901 (8)	3	4		35
	C	61 (29)	415 (22)	972 (11)	12	2	2	52
50 mg oil/g diet	C	50 (29)	322 (13)	919 (7)	0	5	1	17

Data from experiments comparing growth of *Locusta* for different concentrations of wheatgerm oil are given in Table 9 and data for *Schistocerca* are given in Table 10. Clearly the omission of oil severely retarded growth in *Locusta* from the start and few hoppers completed development. Of the three dosages of oil tried, growth was best with the lowest concentration. In contrast, *Schistocerca* initially grew well without oil. But by the time the fifth instar was reached, the average weights of hoppers given 5 and 25 mg of oil per gram of diet exceeded

those of hoppers without oil in all trials, the lower dosage being consistently superior. In all trials the highest dosage (50 mg oil per gram diet) caused a retarded growth rate from the start, and resulted in the lowest percentage of adult emergence. The data of Table 10 show that adult emergence was not much reduced where oil was omitted; however, it was observed that these adults were frequently monstrous and mostly had at least slight wing crumpling.

TABLE 10—GROWTH DATA FOR GROUPS OF *Schistocerca* HOPPERS GIVEN VARIOUS CONCENTRATIONS OF WHEATGERM OIL

Treatment	Exp.	Mean wt. (mg) and numbers of hoppers alive (in brackets) at these times after hatching			Adults obtained			% of larvae at 6 days to become adult
		6 days	18 days	30 days	♂♂	♀♀	Aborts	
No oil	A	77 (20)	518 (11)	1204 (4)	1	1	1	10
	B	93 (9)	555 (7)	1244 (7)	2	3	1	56
	C	69 (22)	523 (13)	1147 (19)	5	1	2	27
	D	74 (54)	464 (42)	1247 (34)	9	6	6	28
5 mg oil/g diet	A	70 (15)	639 (13)	1572 (11)	4	7		73
	B	92 (10)	638 (6)	1415 (16)	3	3		60
	C	68 (33)	489 (25)	1443 (16)	9	5		43
	D	72 (56)	585 (33)	1449 (24)	14	9	2	41
25 mg oil/g diet	A	81 (8)	570 (3)	1449 (3)	2	1		38
	B	87 (10)	490 (10)	1353 (6)	3	3		60
	C	59 (39)	401 (24)	1324 (19)	8	8	2	43
	D	63 (46)	454 (39)	1370 (24)	14	9	2	40
50 mg oil/g diet	A	72 (17)	312 (13)	1044 (9)	0	0	4	None
	B	86 (10)	388 (5)	1347 (2)	0	0		None
	C	57 (31)	254 (27)	1058 (19)	5	4		29
	D	63 (46)	348 (26)	1054 (12)	3	1		12

As with other insects requiring vegetable oils, it was found that wheatgerm oil could be replaced by linoleic acid and α-tocopherol. The individual importance of these two components was examined in experiments using diets without them, or containing them alone and in combination. Data for *Locusta* are given in Table 11 and for *Schistocerca* in Table 12.

Clearly linoleic acid is required for good growth and development in *Locusta*, whereas α-tocopherol, either alone or in combination with linoleic acid, appears to be without effect. A similar situation obtains with *Schistocerca*, though, as was the case with wheatgerm oil, the effects are less marked than with *Locusta*. As before, the numbers of *Schistocerca* adults obtained on the various treatments were of the same order. However, a count of those having some degree of wing crumpling, expressed in Table 12 as a percentage of adults obtained, showed that without linoleic acid the majority (over 90 per cent) came into this category, while the number of abortive final moults was considerable.

TABLE 11—GROWTH DATA FOR GROUPS OF *Locusta* HOPPERS GIVEN DIETS CONTAINING LINOLEIC ACID AND α-TOCOPHEROL

Treatment	Exp.	Mean wt. (mg) and numbers of hoppers alive (in brackets) at these times after hatching			Adults obtained			% of 6-day larvae to become adult
		6 days	18 days	28 days	♂♂	♀♀	Aborts	
No linoleic acid	A	50 (8)	126 (5)	151 (1)	0	0		None
No tocopherol	B	50 (20)	171 (16)	393 (11)	0	1	2	5
	C	51 (20)	125 (13)	301 (4)	0	0		None
No linoleic acid	A	56 (11)	213 (8)	386 (4)	0	1	1	9
0·3 mg/g tocopherol	B	50 (16)	209 (11)	350 (5)	2	0		12
	C	50 (20)	132 (17)	248 (12)	1	0		5
5 mg/g linoleic acid	A	53 (9)	298 (6)	776 (3)	1	2		33
	B	58 (14)	374 (10)	991 (6)	3	3		43
No tocopherol	C	57 (18)	330 (11)	966 (7)	3	4		39
5 mg/g linoleic acid	A	58 (7)	328 (5)	905 (4)	3	1		57
	B	58 (15)	343 (11)	989 (9)	3	6		60
0·3 mg/g tocopherol	C	53 (19)	341 (16)	1013 (14)	4	9	1	69

TABLE 12—GROWTH DATA FOR GROUPS OF *Schistocerca* HOPPERS GIVEN DIETS CONTAINING LINOLEIC ACID AND α-TOCOPHEROL

Treatment	Exp.	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of 6-day hoppers to become adult	% of adults with crumpled wings
		6 days	18 days	28 days	♂♂	♀♀	Aborts		
No linoleic acid	A	53 (16)	430 (13)	1065 (12)	5	3	2	50	93
	B	63 (15)	366 (7)	760 (7)	2	0	3	13	
No tocopherol	C	58 (13)	629 (10)	1489 (5)	2	2		31	
	D	51 (7)	408 (1)	All dead	0	0		None	
No linoleic acid	A	62 (11)	424 (10)	990 (9)	5	1		56	95
	B	63 (15)	445 (7)	1280 (5)	1	3	1	27	
0·3 mg/g tocopherol	C	67 (14)	583 (12)	1383 (9)	4	4	2	57	
	D	54 (12)	406 (4)	1054 (4)	1	1	2	17	
5 mg/g linoleic acid	A	52 (13)	540 (5)	1506 (5)	2	3		38	14
	B	66 (14)	659 (8)	1500 (8)	5	2		50	
No tocopherol	C	58 (15)	720 (5)	1496 (5)	4	1		33	
	D	57 (10)	601 (7)	1374 (6)	4	1	1	50	
5 mg/g linoleic acid	A	60 (14)	586 (8)	1433 (7)	4	3		50	4
	B	69 (16)	591 (12)	1541 (9)	5	4		56	
0·3 mg/g tocopherol	C	68 (14)	717 (7)	1526 (4)	2	2		29	
	D	61 (17)	678 (7)	1638 (5)	2	3		29	

VOL. 4 1960

TABLE 13—GROWTH DATA FOR GROUPS OF *Locusta* HOPPERS GIVEN DIETS CONTAINING VARIOUS FATTY ACIDS

Treatment	Exp.	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained	% of 6-day hopper to become adult
		6 days	18 days	30 days	♂♂ ♀	
No fatty acid	A	32 (25)	68 (14)	All dead	0 0	None
	B	48 (16)	113 (12)	196 (4)	0 0	None
Stearic acid	A	28 (23)	48 (7)	75 (2)	0 0	None
	B	50 (16)	130 (8)	244 (2)	0 0	None
Palmitic acid	A	26 (24)	54 (13)	153 (2)	0 0	None
	B	45 (14)	99 (4)	105 (2)	0 0	None
Myristic acid	A	29 (24)	50 (10)	135 (3)	0 0	None
	B	41 (16)	58 (8)	95 (1)	0 0	None
Oleic acid	A	40 (28)	96 (18)	187 (7)	0 0	None
	B	45 (17)	125 (11)	263 (2)	0 0	None
Linoleic acid	A	43 (25)	197 (17)	692 (10)	7 2	36
	B	57 (19)	310 (15)	816 (8)	5 3	42

TABLE 14—GROWTH DATA FOR GROUPS OF *Schistocerca* HOPPERS GIVEN DIETS CONTAINING VARIOUS FATTY ACIDS

Treatment	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of 6-day hoppers to become adult
	6 days	18 days	30 days	♂♂	♀♀	Aborts	
No fatty acid	50 (11)	268 (6)	591 (3)	0	0		None
Stearic acid	63 (15)	291 (10)	621 (6)	0	1	3	6
Palmitic acid	53 (16)	275 (12)	745 (9)	0	0	7	None
Myristic acid	52 (17)	270 (10)	812 (4)	1	1	1	12
Oleic acid	51 (19)	269 (12)	666 (8)	0	0	3	None
Linoleic acid	61 (12)	358 (6)	1331 (4)	2	1		25

The so-called linoleic acid used in the foregoing experiments (B.D.H. Technical Linoleic acid) is in fact a mixture of fatty acids obtained from linseed oil. According to HILDITCH (1947) the major components of this mixture are linoleic acid (12–27 per cent), linolenic acid (20–62 per cent), and oleic acid (12–41 per cent), while small amounts of palmitic and other saturated acids occur. As purified

linoleic and linolenic acids were not available, their probable importance was demonstrated by the negative method of showing that the various other fatty acids occurring in technical linoleic acid and obtainable in pure form were ineffective in growth trials when compared with technical linoleic acid. α-Tocopherol was included in all the diets used for these experiments, for though it had no effect in combination with crude linoleic acid, this might arguably have been due to its presence in this product. Growth data for experiments using various fatty acids with *Locusta* and *Schistocerca* are given in Tables 13 and 14. Clearly neither the fully saturated acids (stearic, palmitic, myristic) nor the monoethenoid acid (oleic) were effective in replacing technical linoleic acid. It may be presumed, therefore, that the highly unsaturated major components of the crude mixture are essential dietary factors.

The experiments with wheatgerm oil showed that above a certain concentration growth inhibition occurred. An experiment with *Locusta* to determine the optimum amount of crude linoleic acid gave the data shown in Table 15. Optimal

VOL. 4 1960

TABLE 15—GROWTH DATA FOR GROUPS OF *Locusta* HOPPERS GIVEN DIFFERENT CONCENTRATION OF LINOLEIC ACID

Concentration of linoleic acid	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of 6-day hoppers to become adult
	6 days	18 days	24 days	♂♂	♀♀	Aborts	
None (0)	63 (13)	159 (6)	211 (5)	0	0		None
0·5 mg/g diet (× 1)	57 (17)	211 (10)	335 (7)	0	2		12
2 mg/g diet (× 4)	75 (21)	429 (16)	810 (15)	7	7		67
4 mg/g diet (× 8)	67 (17)	425 (11)	955 (9)	2	6		53
8 mg/g diet (× 16)	81 (22)	462 (18)	984 (17)	11	3	1	64
32 mg/g diet (× 64)	62 (14)	329 (8)	523 (5)	0	0	2	None

growth occurred over the range 2–8 mg acid per gram dry diet and inhibition was severe at 32 mg/g. It appeared that the concentration of 5 mg acid per gram diet used in the first experiments with linoleic acid was within the optimal range, and this concentration was maintained in the diets subsequently used in examining sterol requirements.

3. *Cholesterol.* As cholesterol has been found to satisfy the requirements for a dietary sterol shown by all insects hitherto examined, it was included in all diets tried with locusts, though a clear-cut requirement for it could not be demonstrated with the initial yeast-containing diets. This was probably due to the presence of sterols in the yeast and wheatgerm oil contained in these diets. With the development of a yeast-free diet it was possible to demonstrate a sterol requirement for both species of locust. Data from experiments in which wheatgerm oil or cholesterol, or both substances, were omitted from the diet given in Table 1(E) are shown in Tables 16 and 17.

TABLE 16—THE EFFECT OF WHEATGERM OIL AND CHOLESTEROL ON THE GROWTH OF GROUPS OF *Locusta* HOPPERS

Treatment	Exp.	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of 6-day hoppers to become adult
		6 days	18 days	30 days	♂♂	♀♀	Aborts	
No oil	A	35 (1)	All dead		0	0		None
No cholesterol	B	All dead			0	0		None
	C	24 (1)	All dead		0	0		None
5 mg oil/g diet	A	38 (10)	All dead		0	0		None
No cholesterol	B	37 (4)	All dead		0	0		None
	C	24 (14)	All dead		0	0		None
No oil	A	39 (15)	158 (10)	451 (3)	0	1	2	7
5·6 mg chol-	B	42 (13)	166 (9)	351 (5)	1	1		15
esterol/g diet	C	29 (14)	118 (4)	187 (1)	0	0		None
5 mg oil/g diet	A	51 (14)	369 (12)	857 (7)	5	5		71
5·6 mg chol-	B	46 (20)	326 (18)	902 (11)	4	7	1	55
esterol/g diet	C	36 (16)	223 (14)	747 (8)	2	4		38

TABLE 17—THE EFFECT OF WHEATGERM OIL AND CHOLESTEROL ON THE GROWTH OF GROUPS OF *Schistocerca* HOPPERS

Treatment	Exp.	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of hoppers at 6 days to become adult
		6 days	18 days	28 days	♂♂	♀♀	Aborts	
No oil	A	42 (1)	All dead		0	0		None
No cholesterol	B	31 (13)	All dead		0	0		None
	C	50 (10)	All dead		0	0		None
5 mg oil/g diet	A	57 (10)	All dead		0	0		None
No cholesterol	B	43 (15)	113 (3)	All dead	0	0		None
	C	55 (14)	120 (3)	All dead	0	0		None
No oil	A	82 (7)	640 (3)	1284 (3)	3	0		43
5·6 mg chol-	B	64 (15)	487 (13)	1173 (9)	5	1	3	40
esterol/g diet	C	84 (17)	546 (10)	1295 (7)	2	1	6	18
5 mg oil/g diet	A	77 (11)	671 (6)	1505 (5)	2	3		45
5·6 mg chol-	B	56 (16)	482 (14)	1431 (10)	3	4		44
esterol/g diet	C	83 (13)	637 (11)	1480 (7)	3	4		54

Without cholesterol little growth of either *Locusta* or *Schistocerca* occurred. Complete mortality was slightly delayed where wheatgerm oil was included; as the growth effect of oil does not become apparent in the early instars in *Schistocerca* this may perhaps indicate the presence of traces of sterol in oil. The data for diets containing cholesterol but differing in the presence or absence of oil further confirm the conclusions reached on the effect of oil on the growth of both species.

TABLE 18—THE EFFECT OF VARIOUS CONCENTRATIONS OF CHOLESTEROL ON THE GROWTH OF GROUPS OF *Locusta* HOPPERS

Amount of cholesterol	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of 6-day hoppers to become adult
	6 days	10 days	28 days	♂♂	♀♀	Aborts	
0·14 mg/g diet (× 1)	39 (12)	57 (1)	All dead	0	0		None
0·28 mg/g diet (× 2)	40 (13)	All dead		0	0		None
0·42 mg/g diet (× 3)	39 (18)	67 (1)	All dead	0	0		None
0·56 mg/g diet (× 4)	42 (20)	80 (9)	All dead	0	0		None
0·84 mg/g diet (× 6)	57 (16)	125 (15)	846 (11)	5	5		63
1·4 mg/g diet (× 10)	62 (14)	139 (12)	926 (7)	6	1		50
2·3 mg/g diet (× 16)	60 (19)	127 (17)	794 (8)	2	2		21
5·6 mg/g diet (× 40)	58 (19)	110 (19)	989 (14)	4	9	1	68
14·0 mg/g diet (× 100)	57 (16)	114 (14)	900 (1)	0	0		None

TABLE 19—THE EFFECT OF VARIOUS CONCENTRATIONS OF CHOLESTEROL ON THE GROWTH OF GROUPS OF *Schistocerca* HOPPERS

Amount of cholesterol	Mean wt. (mg) of hoppers and numbers alive (in brackets) at these times after hatching			Adults obtained			% of hoppers at 6 days to become adult
	6 days	18 days	30 days	♂♂	♀♀	Aborts	
0·14 mg/g diet (× 1)	46 (13)	113 (5)	All dead	0	0		None
0·28 mg/g diet (× 2)	46 (10)	129 (4)	All dead	0	0		None
0·42 mg/g diet (× 3)	44 (16)	197 (6)	468 (2)	0	0		None
0·56 mg/g diet (× 4)	55 (11)	262 (9)	696 (6)	1	1	2	18
0·84 mg/g diet (× 6)	62 (16)	420 (8)	1477 (8)	3	4		44
1·4 mg/g diet (× 10)	57 (11)	405 (3)	1526 (3)	1	2		27
2·3 mg/g diet (× 16)	57 (11)	400 (3)	1446 (3)	1	2		27
5·6 mg/g diet (× 40)	60 (14)	424 (10)	1592 (7)	4	3		50
14·0 mg/g diet (× 100)	53 (17)	383 (9)	1560 (8)	2	4	2	35

The concentration of cholesterol required for good growth was determined by varying the amount used in a basic diet having the composition given in Table 1(E), but with linoleic acid instead of wheatgerm oil. Growth data for *Locusta* on this diet are given in Table 18, and for *Schistocerca* in Table 19.

VOL. 4 1960

With *Locusta*, little growth occurred for concentrations of 0·5 mg cholesterol per gram diet or less; performances were optimal for concentrations from 0·84 to 5·6 mg/g diet, whereas at 14·0 mg/g growth was retarded and no adults were obtained. *Schistocerca* showed a graded growth response over the range of concentrations 0·14–0·84 mg cholesterol per gram diet and growth was optimal at all concentrations used above 0·84 mg/g, the high concentration of 14·0 mg/g seeming to have little inhibiting effect.

DISCUSSION

Hungry locusts eat diverse substances, a propensity for polyphagy which facilitates the development of artificial diets. On the assumption that protracted feeding requires a biteable food, diets were initially made up in an agar base. However, when *Schistocerca* was found to complete development equally well on amorphous diets lacking agar and with little water, it became clear that the physical form and water content of the food were uncritical.

Though it was apparent that in *Schistocerca* feeding did not depend absolutely on critical chemical or physical factors, it long remained a possibility that palatability of diets might nevertheless be sub-optimal, and thus cause inadequate feeding. Were this so, growth results might occur which could be confused with those due to dietary inadequacy in a strictly nutritional sense. This problem became acute when erratic and contradictory results arose in experiments on the function of carotene. Owing to this, what had at first been interpreted as a nutritional requirement for carotene came to appear more probably an artefact of wide variability in growth on diets which were either unpalatable or nutritionally sub-optimal.

In developing a diet which would give consistent growth while being more synthetic, various hypothetical ways of accounting for poor growth were borne in mind to guide an otherwise trial-and-error approach.

Given satisfactory environmental conditions a food which fails to support growth must be either nutritionally unsuitable or eaten in insufficient amounts. If unsuitable, it may lack essential nutrients, as was the case with all diets tried before a need for ascorbic acid was recognized. Should there be no absolute deficiency, unsuitability may be due to an imbalance in nutrients, a fault which vitiated the first yeast-less diets tried. Again, growth inhibitors might be present; in the light of subsequent work it seems that most of the diets first used must have contained inhibitory amounts of oil.

Unpalatability was envisaged as the likeliest cause of insufficient feeding. Experiments aimed at clarifying this point were indeterminate, but they suggested incidentally that mechanical insufficiency of the gut when dealing with food of inappropriate consistency might also lead to reduced intake. Recognition of this factor was crucial in formulating the diet which finally gave consistent growth results.

Difficulty in assessing dietary defects arose for two reasons. Except in the case of ascorbic acid deficiency, the point at which growth failed was individually

very variable, and after correcting this deficiency some hoppers were able to complete development on most diets used. Secondly, in retrospect, it appears likely that various defects occurred together in varying degree in the earlier diets. Moreover, primary defects may have become secondarily complicated. Vitamin deficiencies may cause loss of appetite, a well-known occurrence in mammalian dietetics, and thus reduce feeding. A particular nutrient may limit the rate of movement of food through the gut, as glucose has been shown to do in the cockroach (TREHERNE, 1957), and consequently, if present in excess, give rise to deficiencies in other components present in concentrations which would otherwise be adequate in a balanced diet. An effect of this sort may well have been operative in the early low cellulose diets, as the increase in cellulose content which so markedly improved growth was made partially at the expense of sugar. If the primary cause of poor growth were sub-optimal feeding, complications might arise from the possibility that in diets prepared infrequently, decay of essential nutrients during storage might proceed at such a rate as to induce nutritional deficiencies during the protracted growth period consequent upon slow feeding.

VOL. 4 1960

This latter situation is more likely where all the diet needed for complete development is dispensed at the start of an experiment, a practice followed in most studies, and necessary where aseptic techniques have been used. Little account seems to have been taken of the possible instability of vitamins during growth trials, although deleterious effects have been attributed to the oxidation of unprotected linoleic acid (FRAENKEL and BLEWETT, 1946) under these circumstances. In the present work decay of nutrients was minimized by replenishment of diet every 2 days during the course of experiments, and by ensuring that stock diets were prepared afresh frequently and stored in a deep freeze.

With the high cellulose diet finally formulated, *Schistocerca* grew reasonably consistently. At the end of larval development mean weights of 1300–1500 mg were obtained, compared with 1500–1700 mg on grass. The percentages of 6-day hoppers to become adult were between 20 and 60; on grass 40–80 per cent survival was usual. Moreover, *Locusta*, which had been at first abandoned owing to its intractability on the early diets, was enabled to complete development with, if anything, rather better survival than *Schistocerca*, though the final mean weights attained (750–1000 mg) were rather lower compared with a grass diet (1200–1400 mg). Thus growth of both species was consistent enough for the use of this diet as a control in examining nutritional requirements. Before discussing these, certain methodological points require consideration.

The control diet was far from optimal. Mortality immediately after emergence was often very high, and in particular, several attempts to bring *Schistocerca* to maturity all failed, although adults kept for 3 months occasionally copulated and eggs were deposited a few times, once in a properly formed pod but otherwise scattered around the cage. Subsequent information showed that the diet was still unbalanced in many respects, but apart from this, growth factors which only manifest themselves during adult development (perhaps through being partially

provided in the egg) may have been lacking. However, the time available precluded extensive work with adults, and in general only criteria based on growth up to emergence were used in assessing the adequacy of diets.

Because it was possible that potential deficiencies might not become manifest during an incomplete period of development, positive information was obtainable only for components of the diet whose omission produced observable effects. Failing such effects, dispensability could not be assumed. This limitation holds theoretically for most nutritional studies made, for only exceptionally have successive generations been successfully reared on synthetic media (VANDERZANT and REISER, 1956), though failure has sometimes not occurred for several generations (WRESSELL, 1955).

In the present case the possibility of potential deficiencies remaining undiscovered was increased by the incompletely synthetic nature of the diet and the non-sterile method of rearing employed, conditions which might, by contributing contaminating micronutrients to the diet, mask real requirements for them.

Failing a totally synthetic diet nothing could be done to ameliorate the first of these drawbacks. It may be remarked that 'syntheticness' is a question of degree, for a 'totally synthetic diet' is probably an ideal abstraction, as many so-called pure substances, especially those prepared from natural materials, contain some impurities (LIPKE and FRAENKEL, 1956). The absence of asepsis constituted a more serious weakness of method. However, lacking a practicable means of rearing locusts aseptically, it was hoped that the use of dry diets would obviate undue development of micro-organisms, and that such contamination as occurred would be minimized by frequent renewal of diet and water-pads. Dry diets used in non-sterile circumstances had provided extensive information on the vitamin requirements of stored products insects (FRAENKEL and BLEWETT, 1943–1950) and in the event proved practicable with locusts.

We must now turn to discuss the idiosyncrasies of diet so far discovered. The requirement for ascorbic acid is most unusual. No insects hitherto examined have needed dietary supplies of this substance although it occurs in the tissues of several (DAY, 1949). Most insects are presumed to synthesize enough for their metabolic requirements, and this has been shown to be so for the cockroach (WOLLMAN *et al.*, 1937). Because of its unusual interest a detailed study of ascorbic acid in *Schistocerca* was made and will be reported at length elsewhere. It was found that on ascorbic-deficient diets the ascorbic acid of the haemolymph, estimated by titration with dichlorophenol–indophenol, was greatly reduced or absent, depending upon the level of ascorbic acid in the diet. In normal grass-fed hoppers the ascorbic acid titre of the haemolymph fluctuates in relation to moulting, being absent or very low immediately after the moult and rising to a high level by mid instar. As a high proportion of deaths on ascorbic-deficient diets occur during moulting, an involvement of ascorbic acid in the mechanism of moulting is indicated.

The lipid requirements for growth of larvae of both *Schistocerca* and *Locusta* are satisfied by a combination of unsaturated fatty acids and cholesterol. Early

work indicated that *Schistocerca* required dietary carotene or vitamin A for complete development, but this was subsequently contradicted. Numerous experiments with various concentrations of carotene show quite clearly that under the experimental conditions it is not required for larval growth. Such a requirement would have been most unusual, for although carotene has been claimed to improve growth and undergo conversion to vitamin A in the silkworm (MURTHY, 1953; YOSHIDA, 1955), most insects studied have needed neither substance. However, though no vitamin A has been detected in locust tissues (GOODWIN, 1952; FISHER and KON, 1955), β-carotene occurs in considerable quantities (if present, as it normally is, in the food), and is involved in several aspects of metabolism. The role of carotene in the complex pigmentation changes associated with phase is well known (GOODWIN, 1952) and it has been shown that without dietary carotene the coloration of gregarious hoppers of *Schistocerca* is abnormal. Not only is there an absence of the yellow colour directly attributable to carotene, but the appearance in hoppers and fledgelings of free, reduced insectorubin, a chemically unrelated pigment, is prevented. The recent demonstration that insectorubin may play some part in oxidative mechanisms in the integument of *Schistocerca* (BELLAMY, 1958) is of interest in this connexion; protein-bound insectorubin occurs without carotene, the effect of which would therefore seem to concern the poising of a tissue milieu such that reduced insectorubin is liberated.

VOL. 4 1960

Carotene seems also to be involved in certain phenomena of reproduction. In the female, eggs accumulate appreciable amounts which are subsequently partially oxidized to astaxanthin during embryogenesis, a conversion that continues throughout larval development (GOODWIN and SRISUKH, 1949). The significance of this is not known, but a relationship between carotenoids and reproductive cells is of quite widespread occurrence (GOODWIN, 1950). In gregarious male locusts, sexual maturation is marked by the development of a vivid yellow colour, caused by the translocation of β-carotene through the hypodermis to the cuticle. Concurrently, in *Schistocerca*, an oil-soluble substance which stimulates specific sexual behaviour in immature females is accumulated in vacuoles in the hypodermis and passed to the surface of the integument (LOHER, 1958). This substance is as yet unidentified, but the production of it in the hypodermis at a time when β-carotene must be in transit is suggestive of an interrelationship between them.

In view of these considerations it would be premature to dismiss carotene as nutritionally inessential, for though apparently without effect on growth from the normal egg to the adult stage, it might be required for more extensive development. As already indicated, the contradictory results obtained in the early experiments with carotene and vitamin A were probably caused by the unreliability of the best diet then available. Nevertheless, there remains the awkward fact that though this early series of experiments eventually resulted in complete development without carotene, in general better growth occurred with it. In attempting to reconcile this with the quite clear dispensability of carotene shown with the later synthetic diets the following points come to mind. Should a potential

carotene deficiency only become manifest at some post-emergence stage of development given optimal growth in other respects, it is feasible that with minimal conditions, as was the case with the yeast-containing diets, the potential deficiency would become manifest at an earlier stage. A situation of this sort was found to occur with respect to ascorbic acid deficiency, which in yeast diets became apparent in the second and third instars, whereas in the later improved synthetic diets growth failed mainly in the fourth instar.

The variability obtained on the yeast diet experiments might then be accounted for perhaps in terms of progressive contamination of the experimental room by diet-derived dust containing carotene (it will be noted that the later experiments in the series tended to produce better growth so far as the carotene-free diets were concerned), or, more probably, in terms of initial variation in the amounts of carotene contained in the eggs. The possibility of producing viable eggs having a low content of carotene from parents on a low carotene artificial food of a type recently described (HOWDEN and HUNTER-JONES, 1959) might help towards a final solution of this problem.

The need for polyunsaturated fatty acids shown by locusts is unusual, for such dietary requirements in insects have hitherto been demonstrated only for certain Lepidoptera. In the beetle, *Tenebrio*, synthesis of linoleic acid has been demonstrated (FRAENKEL and BLEWETT, 1947). Optimal growth of *Blattella* occurred with 3 per cent of corn oil in a dry diet (NOLAND *et al.*, 1949), and lard, or an equivalent mixture of fatty acids, improved growth of *Pseudosarcophaga* (HOUSE and BARLOW, 1956), but in neither case was a requirement for linoleic acid specifically shown. Corn oil was essential for caterpillars of *Pyrausta*, and satisfactory growth occurred when it amounted to 1 per cent of the dry matter of the medium (BECK *et al.*, 1949). Linoleic acid containing 1 per cent of α-tocopherol could replace corn oil (BECK, 1950), but these substances were not tested individually. For those Lepidoptera whose fatty acid requirements are known in detail, vegetable oils have sufficed at concentrations of 2–13 mg/g of dry diet, and have been replaced by pure unsaturated acids in concentrations of 1–6 mg/g (FRAENKEL and BLEWETT, 1946; VANDERZANT *et al.*, 1957). The amounts of oil and crude linoleic acid required by locusts, 5 mg and 2 mg respectively per gram of diet, are of the order needed for these Lepidoptera.

Although α-tocopherol was used with fatty acids for these Lepidoptera, its role is somewhat obscure. With *Ephestia* spp., it functioned mainly in stabilizing linoleic acid, and it could be replaced for this purpose by other anti-oxidants such as gallates or ascorbic acid, but it was additionally considered to have a growth effect *sui generis* (FRAENKEL and BLEWETT, 1946). In contrast, *Pectinophora* required it neither for growth nor for stabilizing the essential fatty acids, although it was retained in the medium apparently as a precautionary stabilizer (VANDERZANT, 1957; VANDERZANT *et al.*, 1957).

Locusts apparently do not require tocopherol, for it was without effect, whether linoleic acid was present or absent. The possibility that it might occur as an impurity in technical linoleic acid was earlier mentioned, but the fact that

Ephestia required tocopherol for B.D.H. technical linoleic acid to be effective in pupal eclosion (FRAENKEL and BLEWETT, 1946) moots against this. It might be argued that a requirement for tocopherol in its anti-oxidant function was masked by the high level of ascorbic acid present in the diet, as this also stabilized linoleic acid for *Ephestia*. However, in *Locusta*, where ascorbic acid deficiency only becomes apparent in the later instars, the omission of linoleic acid affects growth from the start; this suggests that its action is independent of ascorbic acid. It remains possible that had the diet in these experiments been replenished less frequently, tocopherol or other anti-oxidants might have assumed importance in delaying rancidative oxidation. The growth inhibitory effect of excess pure linoleic acid on *Ephestia* was accounted for in these terms, as the diets used were unchanged during the course of experiment (FRAENKEL and BLEWETT, 1946). The inhibition of locust growth by high levels of both oil and linoleic acid was perhaps due to such oxidative products reaching a toxic level rather than to excess linoleic acid itself being toxic.

A characteristic feature of linoleic acid deficiency in both *Ephestia* and *Pectinophora* is the inability of the imago to emerge from the pupa; this constitutes the major cause of mortality in both cases, and has suggested that dietary fatty acids are particularly concerned in the production of moulting fluid. It is of interest to note that in *Schistocerca*, where the absence of linoleic acid affected growth only in the late larval stages, the larval–adult moult was marked by a high abortion rate and, in those that emerged, by a high incidence of wing malformation.

The sterol requirements of locusts are satisfied by concentrations of cholesterol of the order found adequate for other insects. Some, such as *Attagenus* and *Musca vicina*, require as little as 0·1 mg/g dry diet (MCKENNIS, 1947; LEVINSON and BERGMAN, 1955), but optimal dosages are more often in the range 0·5–1·0 mg/g. The concentration of about 0·8 mg/g found optimal for both *Schistocerca* and *Locusta* is comparable to that required by *Blattella* (0·5 mg/g), the most closely related insect previously studied (NOLAND, 1954).

Though essentially similar, the sensitivity of the two species of locusts to lipid deficiencies differ. This is most apparent with linoleic acid, where *Schistocerca* shows little inhibition of growth until the last larval instar, whereas *Locusta* is retarded from the start and rarely reaches the adult stage. Similarly, the effective concentrations of sterol seem more critical in the early growth of *Locusta* than *Schistocerca*. A further difference appears with the highest concentration of cholesterol used; this had little inhibitory effect on *Schistocerca* but retarded the growth of *Locusta* considerably. As the yeast diets initially used contained twice this amount of cholesterol, it appears in retrospect that the failure to rear *Locusta* on them may be accounted for in these terms alone.

SUMMARY

(1) The development of artificial diets on which the locusts *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. could be reared to the adult stage is described.

(2) *Locusta* could not be reared on diets first used which contained yeast. *Schistocerca* could complete larval development on such diets, although growth was poor compared with grass and results were erratic.

(3) With such diets it was shown that ascorbic acid was needed for growth and β-carotene for normal pigmentation in *Schistocerca*.

(4) Yeast was subsequently replaced by ten water-soluble vitamins and a mixture of casein, egg albumen, and peptone. Casein alone was inadequate. The growth of *Schistocerca* on such diets was no better than on yeast-containing diets.

(5) From a study of the amounts of food eaten and the amounts of faeces produced by *Schistocerca* larvae given various artificial diets or grass, it was found that artificial diets had exceptionally high coefficients of digestibility when compared with grass. This suggested that poor growth on them might be a result of insufficient indigestible bulk material. On increasing the proportion of cellulose in diets, growth and survival were notably improved. Such diets were satisfactory for both *Locusta* and *Schistocerca* and provided a suitable basis for the detailed study of nutritional requirements in both species.

(6) The lipid requirements of both species were satisfied by a combination of cholesterol and unsaturated fatty acids. Tocopherol was not required, and although carotene was essential for normal pigmentation in *Schistocerca* its omission from the diet was without effect on the growth of larvae of either species.

Acknowledgements—This work was undertaken for the Anti-Locust Research Centre, London, and was throughout financed by that organization. I am deeply indebted to the past and present directors of the centre, Dr. B. P. UVAROV and Dr. T. H. C. TAYLOR, for their assistance and encouragement in all aspects of the project. The experimental work was carried out in the laboratories of the Department of Zoology and Applied Entomology, Imperial College, London. I wish to thank Professor O. W. RICHARDS for the hospitality of his department, and his staff for much help and discussion. I am grateful to Dr. T. W. GOODWIN of the University of Liverpool for valuable advice on carotenoids in locusts and for the gift of a sample of lycopene, and to Dr. S. K. KON and Dr. L. R. FISHER of the Institute of Dairy Research, Reading, for checking the carotenoid content of *Schistocerca* larvae. Mr. F. RAYMOND of the Grassland Research Institute, Hurley, was kind enough to discuss matters connected with the nutritional value of grass and made available a preparation of crude grass protein. My particular thanks go to Mr. P. HUNTER-JONES and the laboratory staff at the Anti-Locust Research Centre whose never-failing co-operation ensured supplies of locust hatchlings and larvae whenever required; and to Mr. D. MANNING for his help in much of the work.

REFERENCES

BECK S. D. (1950) Nutrition of the European cornborer, *Pyrausta nubilalis* (Hbn.)—II. Some effects of diet on larval growth characteristics. *Physiol. Zool.* **23**, 353–361.

BECK S. D. (1953) Nutrition of the European cornborer, *Pyrausta nubilalis* (Hbn.)—III. An unidentified dietary factor required for larval growth. *J. gen. Physiol.* **36**, 317–325.

BECK S. D., LILLY J. H., and STAUFFER J. F. (1949) Nutrition of the European cornborer, *Pyrausta nubilalis* (Hbn.)—I. Development of a satisfactory purified diet for larval growth. *Ann. ent. Soc. Amer.* **42**, 483–496.

BECK S. D. and STAUFFER J. F. (1957) The European cornborer, *Pyrausta nubilalis* (Hübn.) and its principal host-plant—III. Toxic factors influencing larval establishment. *Ann. ent. Soc. Amer.* **50**, 166–170.

BECKMAN H. F., BRUCKART S. M., and REISER R. (1953) Laboratory culture of the pink bollworm on chemically defined media. *J. econ. Ent.* **46**, 627–630.

BELLAMY D. (1958) The structure and metabolic properties of tissue preparations from *Schistocerca gregaria* (desert locust). *Biochem. J.* **70**, 580–589.

BOTTGER G. T. (1942) Development of synthetic food media for use in nutrition studies of the European cornborer. *J. agric. Res.* **65**, 493–500.

DADD R. H. (1957) Ascorbic acid and carotene in the nutrition of the desert locust, *Schistocerca gregaria* Forsk. *Nature, Lond.* **179**, 427–428.

DAVEY P. M. (1954) Quantities of food eaten by the desert locust, *Schistocerca gregaria* Forsk., in relation to growth. *Bull. ent. Res.* **45**, 539–551.

DAY M. F. (1949) The distribution of ascorbic acid in the tissues of insects. *Aust. J. sci. Res.* (B) **2**, 19–30.

ELLIOTT K. R. (1955) Studies on the nutrition of the Southern armyworm *Prodenia eridania* (Cramer). *Rep. ent. Soc. Ont.* **87**, 17–19.

FISHER L. R. and KON S. K. (1959) Vitamin A in the invertebrates. *Biol. Rev.* **34**, 1–36.

FRAENKEL G. and BLEWETT M. (1943a) The basic food requirements of several insects. *J. exp. Biol.* **20**, 28–34.

FRAENKEL G. and BLEWETT M. (1943b) The vitamin B-complex requirements of several insects. *Biochem. J.* **37**, 687–692.

FRAENKEL G. and BLEWETT M. (1943c) The sterol requirements of several insects. *Biochem. J.* **37**, 692–695.

FRAENKEL G. and BLEWETT M. (1946a) The dietetics of the caterpillars of three *Ephestia* species, *E. kuehniella*, *E. elutella* and *E. cautella*, and of a closely related species, *Plodia interpunctella*. *J. exp. Biol.* **22**, 162–171.

FRAENKEL G. and BLEWETT M. (1946b) Linoleic acid, vitamin E and other fat-soluble substances in the nutrition of certain insects, *Ephestia kuehniella*, *E. elutella*, *E. cautella* and *Plodia interpunctella* (Lep.). *J. exp. Biol.* **22**, 172–190.

FRAENKEL G. and BLEWETT M. (1947) Linoleic acid and arachidonic acid in the metabolism of two insects, *Ephestia kuehniella* (Lep.) and *Tenebrio molitor* (Col.). *Biochem. J.* **41**, 475–478.

FRAENKEL G., BLEWETT M., and COLES M. (1950) The nutrition of the mealworm, *Tenebrio molitor* L. (Tenebrionidae, Coleoptera). *Physiol. Zool.* **23**, 92–108.

FRIEND W. G. (1958) Nutritional requirements of phytophagous insects. *Annu. Rev. Ent.* **3**, 57–74.

GOLBERG L. and DE MEILLON B. (1948a) The nutrition of the larva of *Aedes aegypti* Linnaeus—III. Lipid requirements. *Biochem. J.* **43**, 372–379.

GOLBERG L. and DE MEILLON B. (1948b) The nutrition of the larva of *Aedes aegypti* Linnaeus—IV. Protein and amino acid requirements. *Biochem. J.* **43**, 379–387.

GOLBERG L., DE MEILLON B., and LAVOIPIERRE M. (1945) The nutrition of the larva of *Aedes aegypti* L.—II. Essential water-soluble factors from yeast. *J. exp. Biol.* **21**, 90–96.

GOODWIN T. W. (1949) The biochemistry of locusts—II. Carotenoid distribution in solitary and gregarious phases of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) and the desert locust (*Schistocerca gregaria* Forsk.). *Biochem. J.* **45**, 472–479.

GOODWIN T. W. (1950) Carotenoids and reproduction. *Biol. Rev.* **25**, 391–413.

GOODWIN T. W. (1952) The biochemistry of locust pigmentation. *Biol. Rev.* **27**, 439–460.

GOODWIN T. W. and SRISUKH S. (1949) The biochemistry of locusts—I. The carotenoids of the integument of two locust species (*Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.). *Biochem. J.* **45**, 263–268.

VOL. 4 1960

GUNN D. L. and HUNTER-JONES P. (1952) Laboratory experiments on phase differences in locusts. *Anti-Locust Bull.* **12**, 1–37.

HAMILTON A. G. (1950) Further studies on the relation of humidity and temperature to the development of two species of African locusts—*Locusta migratoria migratorioides* (R. and F.), and *Schistocerca gregaria* (Forsk.). *Trans. R. ent. Soc. Lond.* **101**, 1–58.

HILDITCH T. P. (1947) *The Chemical Constitution of Natural Fats.* Chapman and Hall, London.

HINTON H. E. (1956) Dietary requirements of insects. Amino acids and vitamins. *Sci. Progr.* **44**, 292–309.

HOUSE H. L. and BARLOW J. S. (1956) Nutritional studies with *Pseudosarcophaga affinis* (Fall), a dipterous parasite of the spruce budworm, *Choristoneura fumerifana* (Clem.)—V. Effects of various concentrations of the amino acid mixture, dextrose, potassium ion, the salt mixture, and lard on growth and development; and a substitute for lard. *Canad. J. Zool.* **34**, 182–189.

HOWDEN G. F. and HUNTER-JONES P. (1958) An artificial diet for the laboratory rearing of locusts. *Nature, Lond.* **182**, 1527–1528.

HUSAIN M. A., MATHUR C. B., and ROONWAL M. L. (1949) Studies on *Schistocerca gregaria* (Forskål)—XIII. Food and feeding habits of the desert locust. *Indian J. Ent.* **8**, 141–163 (1946).

ISHII S. (1952) Some problems on the rearing method of rice stem borer by synthetic media under aseptic conditions. *Oyo-Kontyu* **8**, 93–98.

LEVINSON Z. H. (1955) Nutritional requirements of insects. *Riv. Parassit.* **16**, 113–183.

LEVINSON Z. H. and BERGMAN E. D. (1955) Steroid utilisation and fatty acid synthesis by the larva of the housefly, *Musca vicina*. *Biochem. J.* **65**, 254–260.

LIPKE H. and FRAENKEL G. (1956) Insect nutrition. *Annu. Rev. Ent.* **1**, 17–44.

LOHER W. (1958) An olfactory response of immature adults of the Desert Locust. *Nature, Lond.* **181**, 280.

MCKENNIS H. (1947) The growth-promoting activity of cholesterol and related compounds in black carpet beetle larvae. *J. biol. Chem.* **167**, 645–654.

MATSUMOTO Y. and YASUE Y. (1953) Aseptic rearing of the oriental fruit moth, *Laspeyresia molesta*, Busck, by synthetic media. *Ohara Inst. Agr. Res. Spec. Rept.* **40**, 205–208.

MURTHY M. R. V. (1953) *Studies on the Nutrition of the Silkworm*, Bombyx mori *L.* Doctoral thesis, University of Bangalore, India.

NOLAND J. L. (1954) Sterol metabolism in insects—I. Utilisation of cholesterol derivatives by the cockroach, *Blattella germanica* L. *Arch. Biochem. Biophys.* **48**, 370–379.

NOLAND J. L., LILLY J. H., and BAUMANN C. A. (1949) Vitamin requirements of the cockroach *Blattella germanica* (L.). *Ann. ent. Soc. Amer.* **42**, 154–164.

RAYMOND W. F. and TILLEY J. M. A. (1956) The extraction of protein concentrates from leaves. *Colon. Pl. Anim. Prod.* **6**, 3–19.

SINGH K. R. P. and BROWN A. W. A. (1957) Nutritional requirements of *Aedes aegypti* L. *J. Ins. Physiol.* **1**, 199–220.

SUBAROW Y. and TRAGER W. (1940) The chemical nature of growth factors required by mosquito larvae—II. Pantothenic acid and vitamin B_6. *J. gen. Physiol.* **23**, 561–568.

THORSTEINSON A. J. (1953) The chemotactic responses that determine host specificity in an oligophagous insect (*Plutella maculipennis* (Curt.), Lepidoptera). *Canad. J. Zool.* **31**, 52–72.

THORSTEINSON A. J. (1958) The chemotactic influence of plant constituents on feeding by phytophagous insects. *Ent. exp. appl.* **1**, 23–27.

TRAGER W. (1948) Biotin and fat-soluble materials with biotin activity in the nutrition of mosquito larvae. *J. biol. Chem.* **176**, 1211–1223.

TRAGER W. (1953) Nutrition. In: ROEDER K. D. (ed.) *Insect Physiology.* Chapman and Hall, London.

TRAGER W. and SUBAROW Y. (1938) The chemical nature of growth factors required by mosquito larvae—I. Riboflavin and thiamin. *Biol. Bull., Woods Hole* **75**, 75–84.

TREHERNE J. E. (1957) Glucose absorption in the cockroach. *J. exp. Biol.* **34**, 478–485.

VANDERZANT E. S. (1957) Growth and reproduction of the pink bollworm on an amino acid medium. *J. econ. Ent.* **50**, 219–221.

VANDERZANT E. S., KERUR D., and REISER R. (1957) The role of dietary fatty acids in the development of the pink bollworm. *J. econ. Ent.* **50**, 606–608.

VANDERZANT E. S. and REISER R. (1956a) Aseptic rearing of the pink bollworm on synthetic media. *J. econ. Ent.* **49**, 7–10.

VANDERZANT E. S. and REISER R. (1956b) Studies of the nutrition of the pink bollworm using purified casein media. *J. econ. Ent.* **49**, 454–458.

WILDE J. DE (1958) Host-plant selection in the Colorado beetle larva. *Ent. exp. appl.* **1**, 14–22.

WOLLMAN E., GIROUD A., and RATSIMAMANGA R. (1937) Synthèse de la vitamine C chez un insecte orthoptère (*Blattella germanica*) en élevage aseptique. *C.R. Soc. Biol., Paris* **124**, 434–435.

WRESSALL H. B. (1955) Rearing the European corn borer, *Pyrausta nubilalis* (Hbn.) (Lepidoptera: Pyralididae) on an artificial diet. *Rep. ent. Soc. Ont.* **86**, 10–13.

YOSHIDA T. (1955) *Bull. seric. Exp. Sta. Japan* **14**, 351–426 (not seen).

VOL.
4
1960

J. Ins. Physiol., 1960, *Vol.* 4, *pp.* 348 *to* 349. *Pergamon Press Ltd., London. Printed in Great Britain*

OXIDATIVE PHOSPHORYLATION IN *PHORMIA REGINA* LARVAE*

R. W. NEWBURGH,† L. N. POTTER, and V. H. CHELDELIN

Science Research Institute and Department of Chemistry,
Oregon State College, Corvallis, Oregon

(*Received* 22 *March* 1960)

Abstract—Oxidative phosphorylation was shown to occur in the mitochondria isolated from *Phormia regina* larvae. The P/O ratios obtained were similar to adult preparations. In addition, these reactions were shown to be catalysed by β-OH butyrate.

Oxidative phosphorylation reactions in preparations from the thoracic muscle of adult *Phormia regina* have been well documented (SACKTOR, 1953; LEWIS and SLATER, 1954). In contrast to the information available on adult preparations, little is known concerning the presence of these reactions in the larvae of the blowfly. Oxidative phosphorylation was shown to occur in the larvae of this insect. Of particular interest was the fact that this process occurred in the presence of β-OH butyrate contrary to published results with adult preparations.

MATERIALS AND METHODS

THE larvae were grown under sterile conditions as reported previously (MCGINNIS, NEWBURGH, and CHELDELIN, 1956). They were removed from the growth medium and washed three times with distilled water prior to homogenization. Cell-free preparations from 3-day-old larvae were prepared by homogenizing in a Dounce homogenizer. It was necessary to use a Waring blendor for older larvae (i.e. 6-day) because of the heavier cuticle. Various grinding media were tried such as 0·25 M sucrose; 0·25 M sucrose–0·001 M Versene; 0·9 per cent KCl–0·001 M Versene; but little difference in activity occurred. The addition of albumin also had little or no effect.

RESULTS AND DISCUSSION

The results of several representative experiments are shown in Table 1. It is interesting to note that the oxidation of β-OH butyrate results in phosphorylation since this has not been reported for adult preparations. This finding may be of particular significance since the bulk of the lipids are synthesized (HODGSON, CHELDELIN, and NEWBURGH, 1960) during larval growth and thus these animals might serve as a suitable source to study the relationships that exist between synthetic and degradative processes involving lipids. Similar to other systems, the reaction was inhibited by antimycin A and dinitrophenol.

* Supported by grants-in-aid from the Research Corporation, and Division of Research Grants, U.S. Public Health Service and the National Science Foundation. Published with approval of the Monographs Publication Committee, Research Paper No. 377, School of Science, Department of Chemistry.

† Scholar of Ethel M. Craig Memorial Grant from the American Cancer Society.

TABLE 1—OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA PREPARATIONS FROM LARVAE OF *P. regina*

Age of larvae (days)	Substrate	μ atoms O_2 consumed	μM phosphorus esterified	P/O
3	Succinate	14·7	31·5	2·1
	Pyruvate + Malate	7·7	22·4	2·9
	β-OH butyrate	1·9	3·5	1·8
	α-Glycerol-PO_4	2·5	3·5	1·4
	Succinate + Albumin	17·5	32·9	1·9
6	Succinate	5·8	11·9	2·0
	Pyruvate + Malate	4·8	13·6	2·8
	β-OH butyrate	2·3	3·5	1·5
	α-Glycerol-PO_4	1·4	3·4	2·4

Each flask contained: 40 μM phosphate buffer, pH 7·2; 5 μM ADP; 100 μM glucose; 10 μM $MgCl_2$; 70 Kunitz–McDowell Units of hexokinase; 0·2 μM DPN and TPN; 20 μM of substrates except malate which was 3 μM. Total volume 3·0 ml. Temperature 30°C. Time, 30 min.

VOL. 4 1960

REFERENCES

HODGSON E., CHELDELIN V. H., and NEWBURGH R. W. (1960) Phospholipid patterns in *Phormia regina* (Meig.). *J. biol. Chem.* In press.

LEWIS S. E. and SLATER E. C. (1954) Oxidative phosphorylation in insect sarcosomes. *Biochem. J.* **58**, 207–217.

MCGINNIS A. J., NEWBURGH R. W., and CHELDELIN V. H. (1956) Nutritional studies on the blowfly, *Phormia regina* (Meig.). *J. Nutr.* **58**, 309–324.

SACKTOR B. (1953) Investigations on the mitochondria of the housefly, *Musca domestica* L.—III. Requirements for oxidative phosphorylation. *J. gen. Physiol.* **37**, 343–359.

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

K. U. CLARKE: Studies on the relationships between air temperature and the internal body temperature of *Locusta migratoria*

H. CHINO: Enzymatic pathways in the formation of sorbitol and glycerol in the diapausing egg of the silkworm, *Bombyx mori*—I. On the polyol dehydrogenases

L. Y. FINLAYSON: A comparative study of the effects of denervation on the abdominal muscles of saturniid moths during pupation

R. H. HACKMAN and M. GOLDBERG: Composition of the oothecae of three Orthoptera

D. S. ANDERSON: The respiratory system of the egg-shell of *Calliphora erythrocephala*

W. H. TELFER and C. M. WILLIAMS: The effects of diapause, development, and injury on the incorporation of radioactive glycine into the blood proteins of the Cecropia silkworm

M. V. BRIAN and C. A. H. CARR: The influence of the queen on brood rearing in ants of the genus *Myrmica*

K. U. CLARKE and R. W. BALDWIN: The effect of insect hormones and of 2 : 4-dinitrophenol on the mitochondrion of *Locusta migratoria* L.

G. BAKER, J. H. PEPPER, L. H. JOHNSON, and E. HASTINGS: Estimation of the composition of the cuticular wax of the mormon cricket, *Anabrus simplex* Hald.

T. ITO: Effect of sugars on feeding of larvae of the silkworm, *Bombyx mori* L.

L. BARTON BROWNE: The rôle of olfaction in the stimulation of oviposition in the blowfly, *Phormia regina*

W. HANEC and S. D. BECK: Cold hardiness in the European corn borer, *Pyrausta nubilalis*

M. LÜSCHER und F. ENGELMANN: Histologische und experimentelle Untersuchungen über die Auslösung der Metamorphose bei *Leucophaea maderae*

H. E. HINTON: Cryptobiosis in the larva of *Polypedilum vanderplanki* Hint. (Chironomidae)

R. H. DADD: The nutritional requirements of locusts—II. Utilization of sterols

J. S. BARLOW and H. L. HOUSE: Effects of dietary glucose on haemolymph carbohydrates of *Agria affinis* (Fall.)

K. N. MEHROTRA: Development of the cholinergic system in insect eggs

M. P. BLAUSTEIN and H. A. SCHNEIDERMAN: A brief survey of the effects of potential antimetabolites and enzymes on the development of giant silkmoths

VOL.
4
1960